Phenotypic characterization of B cells and serum cytokine responses during Tuberculosis and Type 2 Diabetes

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DECLARATION

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Ayanda Noncedo Shabangu December 2019

ABSTRACT

Tuberculosis (TB) remains a world pandemic, claiming the lives of 1.7 million people in 2017. Several risk factors have been identified as key attributes to the development of TB, such as type 2 diabetes (T2D). Cellular function of immune cell types are altered in TB-T2D patients compared to TB patients, leaving the host vulnerable to increased *Mycobacterium tuberculosis* (*Mtb*) replication and enhanced disease severity. Emphasis has been placed on the role of T-cells and macrophages in TB-T2D comorbidity, however, B-cells also play a fundamental role in the adaptive immune response to TB. Since limited information is available on the frequency of B cells in TB in the context of T2D, we aimed to determine the frequencies of regulatory and killer B-cell, through the expression of the cell surface markers, FasL (CD178+) and IL-5Rα (CD125+). In addition, we investigate imbalances in host immune markers in healthy controls, T2D patients and TB patients with and without T2D. The immunological responses, including regulatory, memory and apoptotic B-cell function, are impaired in T2D, TB and TB-T2D patients at the initiation of TB treatment, and improve in TB and TB-T2D patients following two months into anti-TB treatment. In addition, markers associated with cell proliferation, cell development, chemotactic function, granuloma formation and monocyte recruitment are impaired, highlighting the need for effective T2D management in combating TB.

OPSOMMING

Tuberkulose (TB) bly 'n wêreldpandemie wat die lewens van 1,7 miljoen mense in 2017 geëis het. Verskeie risikofaktore is geïdentifiseer as sleutelkenmerke vir die ontwikkeling van TB, soos tipe 2-diabetes (T2D). Die sellulêre funksie van immuunselle in TB-T2D-pasiënte is anders in vergelyking met TB-pasiënte, wat die gasheer kwesbaar maak vir verhoogde *Mycobacterium tuberculosis* (*Mtb*) replikasie en die erns van die siekte verhoog. Klem is gelê op die rol van T-selle en makrofage in die TB-T2D mede-morbiditeit. B-selle speel egter ook 'n fundamentele rol in die aanpasbare immuunrespons op TB. Aangesien beperkte inligting beskikbaar is oor die frekwensie van B-selle in TB in die konteks van T2D, het ons ten doel gehad om die frekwensies van regulatoriese en moordenaar B-selle te bepaal, deur die uitdrukking van die seloppervlakte-merkers, FasL (CD178+) en IL-5Rα (CD125+) te meet. Daarbenewens ondersoek ons wanbalanse in die gasheer immuunmerkers in gesonde kontroles, T2D-pasiënte en TB-pasiënte met en sonder T2D. Die immunologiese reaksies, insluitend regulatoriese, geheue en apoptotiese B-selfunksie, word aangetas in T2D-, TB- en TB-T2D-pasiënte voor die aanvang van TB behandeling, en verbeter in die TB- en TB-T2D-pasiënte na twee maande van TB behandeling. Verder word merkers wat verband hou met selproliferasie, selontwikkeling, chemotaktiese funksie, granuloomvorming en monosiet-werwing benadeel, wat die behoefte aan effektiewe T2D-bestuur in die bestryding van TB benadruk.

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Abbreviations

AGEs - Advanced glycation end products

APCs – Antigen presenting cells

BCR – B cell receptor

Breg – Regulatory B cells

CD – Cluster Differentiation

CRP – C-reactive protein

DCs – Dendritic cells

ER – Endoplasmic reticulum

FFA – Free fatty acids

GLUT – Glucose transporters

HbA1c – Haemoglobin A1c

HC - Healthy controls

IDF – International Diabetes Foundation

IFN-γ - Interferon gamma

IL- Interleukin

iNOS -Iinducible nitric oxide synthase

ITAMs - Immune receptor tyrosine-based activation motifs

MAPKs – Mitogen-activated protein kinases

MHC – Major histocompatibility complex

Mtb – Mycobacterium tuberculosis

NF-kappa B – Nuclear factor kappa B

NK – Natural killer cells

NOD – Nucleotide-binding oligomerization

PAMPs – Pathogen-associated molecular patterns

PPR – Pathogen recognition receptor

RBC – Red blood cells

ROS – Reactive oxidative species

T2D – Type 2 diabetes

TB - Tuberculosis

TB-T2D – TB patients with type 2 diabetes

TCR – T cell receptor

TGF-β - Transforming growth factor-β

Th – T helper cells

TLR – Toll like receptor

TNF-α - Tumor necrosis factor alpha

WHO – World Health Organization

Chapter 1

1. Tuberculosis

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis (Mtb)*, a bacterium that has infected 1.7 billion people in the world according to the 2018 World Health Organization (WHO) global TB report. It is reported that only 5-10% of infected individuals will develop active TB disease in their lifetime (WHO global TB report, 2018). This is, however, still problematic as 10 million people are affected by this disease annually, claiming the lives of more than 1.3 million people yearly. TB incidence rates are the highest on the African and Asian continents in comparison to other continents on the globe with Sub-Saharan Africa and South East Asia mostly impacted (Figure 1.1). In efforts to eradicate the TB pandemic, the WHO has called for zero TB by 2035. Researchers reacted to this call for action and have made great strides towards improved diagnostic tools and treatment regimens. However, the immune response against *Mtb* still remains partially understood.

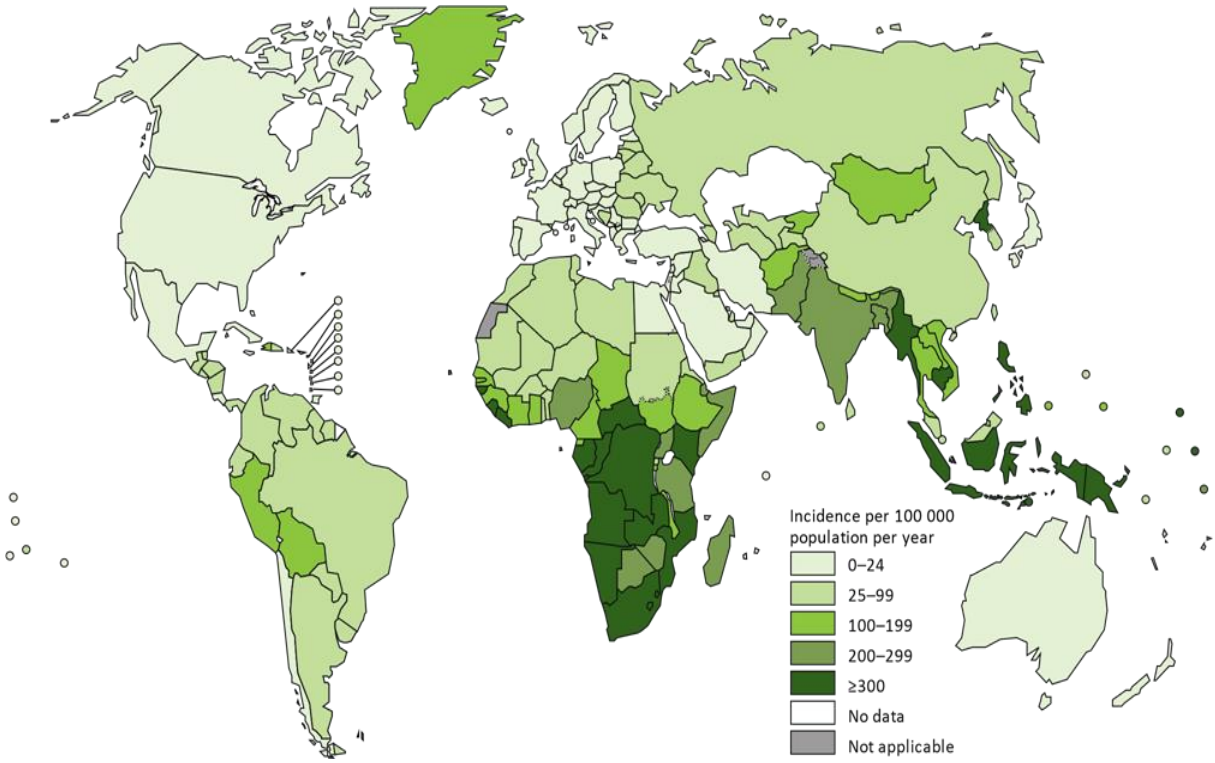

***Figure 1. 1*: The Global incidence rates reported in East Asia region with more than 300 persons per 100 000 per year contract TB**. (WHO Global Tuberculosis Report, 2018).

The transmission of *Mtb* begins with the inhalation of an *Mtb* containing droplet expectorated by individuals with TB. The bacteria travels to the alveoli where they encounter large phagocytes known as alveolar macrophages (Cohen et al., 2018). The recognition of *Mtb* by phagocytic cells is essential for the activation of phagocytic cells and initiation of the innate immune response (Cohen et al., 2018) This is achieved through the interaction of the mycobacteria with Toll-like receptors (TLRs). TLRs are a type of pathogen recognition receptors (PRRs) expressed on various innate immune cells such as macrophages, dendritic cells (DCs), natural killer (NK) cells and cells of the adaptive immune response including B and T lymphocytes (Reviewed by Janssens and Beyaert, 2003). Other PRRs include nucleotide-binding oligomerization domain (NOD) Like Receptors, C-Type Lectins and Dectin-1. They recognize highly conserved structural motifs shared by pathogens known as pathogen-associated molecular patterns (PAMPs). The signalling of TLRs occurs through the induction of the adaptor molecular MyD88, which is responsible for the activation of the mitogen-activated protein kinase (MAPKs) and nuclear factor kappa B (NF-κB) pathways (Hemmi et al., 2000). In the presence of *Mtb* these pathways induce the production of inflammatory cytokines and upregulate co-stimulatory molecules of antigen presenting cells (APCs) (Blander and Medzhitov, 2004; Li et al., 2009).

Antigen presenting cells express major histocompatibility complex (MHC) class II on their cell surface and these immune cells are phagocytes; macrophages, DCs and antibody producing B cells. *Mtb* bacilli are taken up by APCs, degraded in the endocytic pathway and presented on MHC class II molecules on the surface of the APCs. Macrophages and DCs recognize and internalize the pathogen through phagocytosis. Inside the phagosome there are lysosomes with antimicrobial enzymes, degradative peptides and proteases, which degrade the pathogen into peptides for antigen presentation (Arcos et al., 2017). *Mtb* is however able to prevent the fusion of the lysosome and phagosome to increase its survival in the cells (Sundaramurthy et al., 2017).

Alveolar macrophages are the first APCs *Mtb* encounter in the alveoli, their primary objective is to clear *Mtb*, however, some bacilli are persistent and can survive and replicate inside the macrophages. In efforts to eradicate *Mtb,* infected alveolar macrophages produce cytokines and recruit other immune cells like neutrophils, NK cells, monocyte derived macrophages, DCs and T cells to the site of disease causing

tissue remodelling and inflammation in the lung that results in the formation of granulomas (Volkman et al., 2010). The granuloma is a complex of immune cells where the exterior cuff consists of lymphocytes and the core of epithelioid macrophages and multinucleated giant cells. Should the bacteria persist, the centre becomes necrotic and rich in lipid content (Marakalala et al., 2016), which is utilized by *Mtb* as a carbon source (Menon et al., 2019) aiding their survival and replication in the lung. Subsequently, necrotic granulomas can rupture into the airways that leads to the spread of *Mtb* within the lung and to other organs.

The recognition of *Mtb* infected macrophages by T cells is essential for the containment of the mycobacteria. However, in the presence of *Mtb*, T cell recruitment and responses are delayed, promoting the survival of persistent *Mtb* in the lung (Chackerian et al., 2002). T helper 1 (Th1) cells, a subset of $CD4^+$ T cells, activate macrophages through the production of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), inducing apoptosis of the *Mtb* infected cells and inflammation (Lyadova and Panteleev, 2015; Sallin et al., 2017). TNF-α is involved in systemic inflammation and enhances the migration of immune cells to the site of infection (Butler et al., 2017). IFN-γ, produced by Th1 cells, does not only enhance the microbicidal activity of macrophages but also the expression of inducible nitric oxide synthase (iNOS) intermediates (Tötemeyer et al., 2006; Braverman and Stanley, 2017). This highlights the role of cytokines in coordinating an immune response by initiating the recruitment and activation of the immune cells to the *Mtb* infected areas of the lung.

2. Type 2 Diabetes

Type 2 diabetes (T2D) is a metabolic disorder characterized by high blood glucose and insulin resistance. The prevalence of T2D is on a constant rise in low- and middle-income countries, due to the reduced intake of nutritional foods and lack of physical activity. It is also heavily correlated to social-economic factors found in low- and middle-income countries with people living in these countries having an increased risk of developing T2D (Remais et al., 2013). According to the International Diabetes Foundation (IDF) 2017 report, the number of people living with T2D will increase with 156% in Sub-Saharan Africa by 2045 (Figure 1.2). In no other region in the world is the increase in diabetes prevalence this high. Alarmingly, 66.7% of the Sub-Saharan

population are currently not aware of their T2D status and therefore remain undiagnosed (IDF 2017).

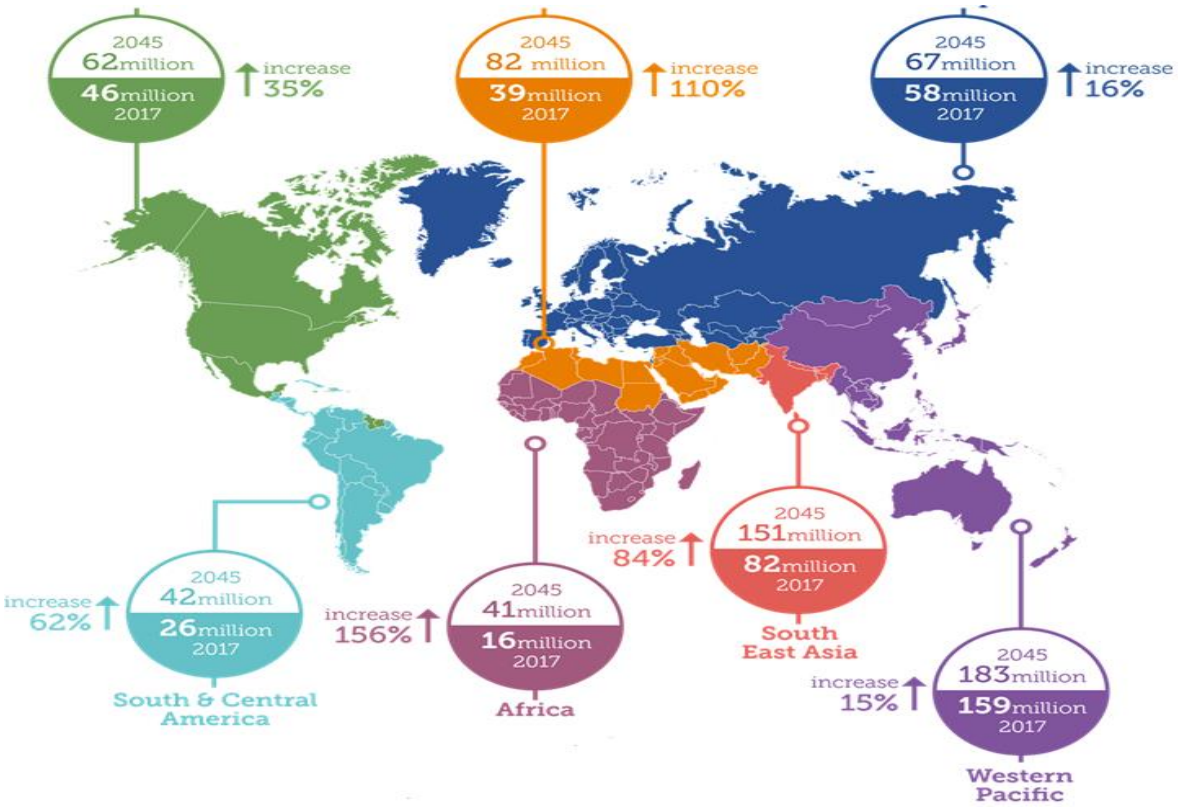

Figure 1. 2: Estimated increases in global diabetes prevalence. The graph highlights the prevalence of diabetes in the different regions of the world. In 2017, the Western Pacific region had the highest number of people living with diabetes; yet, by 2045 the greatest increase in people living with diabetes will be seen in the Sub-Saharan African region. More than a 2-fold increase in the numbers of patients with diabetes, the highest globally, will be seen in this region (IDF diabetes atlas - 2017 Atlas).

Glucose homeostasis is regulated by metabolic pathways, gluconeogenesis and glycogenolysis. The former is responsible for the generation and storage of glucose and the latter for the breakdown of glycogen. These metabolic pathways are responsible for the maintenance of blood glucose in the body. Elevated blood glucose signals the pancreatic β cells to release insulin (Tomás et al., 2002). Insulin, in turn, regulates the absorption of glucose in the liver, fat tissue and skeletal muscle cells (Tomás et al., 2002). The uptake of glucose in these tissues is regulated by specific glucose transporters with GLUT-1 and GLUT-4 being two distinct glucose transporters in muscle tissue (Barnard and Youngren, 1992). During hyperglycaemia, the binding of insulin to the insulin receptor upregulates the PI3K/AKT signalling cascade resulting in the increased expression of GLUT4 and downregulation of the gluconeogenesis pathway (Huang et al., 2018; Watson and Pessin, 2006). GLUT4 then translocates to the cell membrane of muscle tissues allowing for the uptake of glucose and by doing

so decreasing the blood glucose levels (Watson and Pessin, 2006). Hyperglycaemia observed during T2D leads to an increase in β cell activity, enhancing cell mass and insulin secretion due to the high demand for insulin production.

T2D is associated with obesity and low-grade chronic inflammation with increased expression of pro-inflammatory cytokines and adipokines such as interleukin-1 beta (IL-1β), IL-6, TNFα and leptin (Reviewed by Wieser et al., 2013). Systemic C-reactive protein (CRP) levels for example are directly correlated with a higher HbA1c in T2D (King et al., 2003). Metabolic stresses such oxidative stress and endoplasmic reticulum (ER) stress promote inflammation resulting in the development of insulin resistance. In T2D oxidative stress is induced through the production of reactive oxygen species (ROS) and the decreased levels of antioxidative enzymes in impaired β cells leaves the host vulnerable to oxidative stress (Evans et al., 2003). In the presence of hyperglycaemia there is a drastic demand for insulin production and insulin influx through the ER of β cells induces ER stress (Scheuner et al., 2005). Other factors contributing to the inflammation observed include free fatty acids (FFA) and advanced glycation end products (AGEs), which are elevated in T2D patients. They bind to specific cell receptors such as TLRs and RAGE (Syed et al., 2018).

Obesity is a major risk factor for T2D and contributes to the increase in basal cytokine levels and chronic inflammation in T2D. The secretion of cytokines and chemokines from the adipose tissue into the blood stream promotes inflammation in other tissues such as the skeleton muscle. Cytokines produced by the adipose tissue, causes the migration of immune cells into the adipose tissue, inducing the phenotypic switching of macrophages into pro-inflammatory M1 macrophages (Fujisaka et al., 2009; Lumeng et al., 2007). The dysregulated immune response associated with T2D is also apparent in the chronic activation of T cells due to the hyperglycaemia (Richard et al., 2017) and in an attempt to maintain normal physiological cytokine responses. The altered adaptive immune responses and chronic inflammation present in obese T2D is correlated with increased susceptibility respiratory infections such as *Streptococcus pneumoniae* (Martinez et al., 2014), *klebsiella pneumoniae* (Martinez et al., 2016) and *Mtb* (Jeon and Murray, 2008).

3. Tuberculosis and Type 2 Diabetes

T2D is a risk factor for TB (Jeon and Murray, 2008). Individuals who are latently infected with *Mtb* have a 3 to 8-fold increased chance of progressing from latent infection to active TB disease (Restrepo et al., 2011). This is a major concern as it is estimated that a quarter of the world's population is latently infected with *Mtb* with most of these individuals living in countries with an estimated increase in T2D prevalence (Figure 1.1 and Figure 1.2) (Getahun et al., 2015; Houben and Dodd, 2016; WHO, Tuberculosis, 2018).

T2D patients with poorly controlled hyperglycaemia (HbA1c greater than 8%) have an even greater risk of progressing to active TB disease in comparison to T2D patients with controlled glycaemia (Vallerskog et al., 2010). When patients with T2D develop TB, they have a higher bacterial load and there is a delay in their sputum culture conversion two months into TB treatment in comparison to TB patients without T2D (Jeon and Murray, 2008a; Riza et al., 2016; Stevenson et al., 2007). They are also more likely to fail TB treatment, experience recurrent disease and have a higher mortality rate in comparison to TB patients without T2D (Dooley et al., 2009).

3.1 T cells

T cells originate from the bone marrow and migrate to the thymus where maturation is completed. The maturated T lymphocytes enter the bloodstream and the peripheral lymphoid organs where they encounter antigens presented by MHC II molecules. The T cell receptor (TCR) exists in two forms with 95% of T cells containing the alpha and beta (αβ) subunit while the remaining 5 % have the gamma and delta (γδ) subunit (Andreu-Ballester et al., 2013). CD4 and CD8 are co-receptors that are bound at distinct regions on the MHC class II and MHC class I molecules, respectively. Binding of the co-receptors brings the cytoplasmic tails of these receptors in close proximity to the cytoplasmic tail of the TCR containing the immune receptor tyrosine-based activation motifs (ITAMs). The co-receptor linked tyrosine kinase, Lck, phosphorylates the ITAM initiating the TCR signalling cascade (Gaud et al., 2018). Therefore, in order for T cells to recognize *Mtb* peptides and to become activated, it requires the presentation of peptides to MHC class II molecules and the interaction between the co-receptors and MHC class II molecules.

When T cells encounter *Mtb* they are activated and polarize into effector and memory T cells. Memory immune cells evoke a rapid reactivation of effector cells, increased immune cell proliferation and they evoke a rapid adaptive immune response. However, pathogen persistence and chronic inflammation can result to T cell exhaustion. Persist antigen exposure over a long period of time alters memory T cell functionality (Han et al., 2010). During T cell exhaustion, the cytokines secreted by effector T cells is suppressed and T cell proliferation is reduced (Day et al., 2014). A similar trend is also evident in TB-T2D patients were memory T cells are increased and the frequency of the naïve and effector T cells are decreased (Kumar et al., 2016).

3.2 B cells

B cells are a subset of leukocytes derived from stem cells in the bone marrow. The multipotent stem cells are found in the bone marrow differentiate into lymphoid and then into B cell progenitors (Zou et al., 2017). After maturation, B lymphocytes enter circulation and encounter antigens in the peripheral lymphoid organs. Once activated, B cells undergo B cell differentiation and clonal expansion (Figure 1.3A). The principal of clonal expansion is for activated B cells to express antigen specific receptors with an increased binding affinity and specificity as the parent antigen.

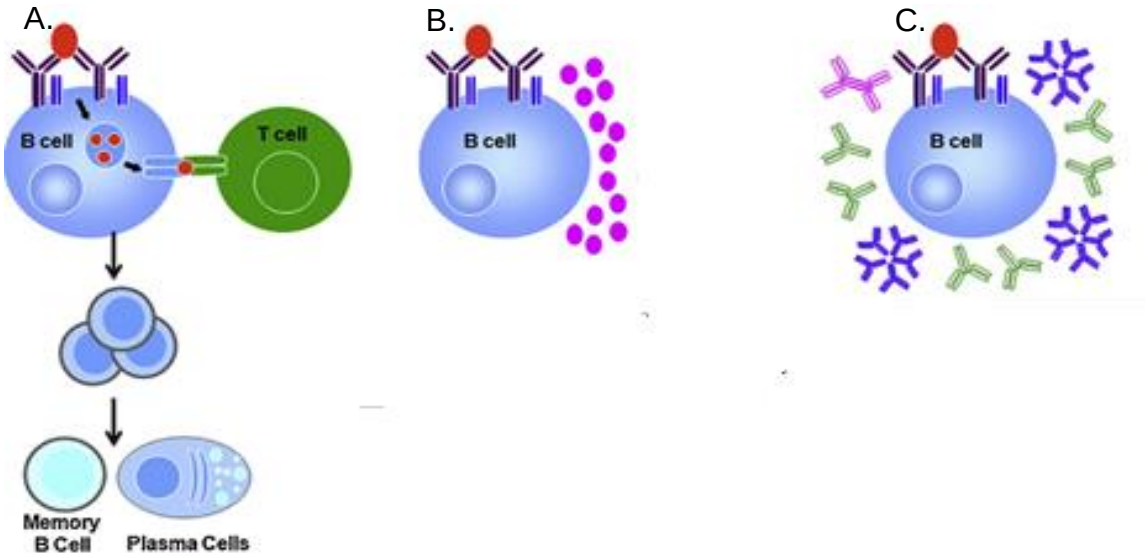

Figure 1. 3: The immunological mechanisms of B cells. *A) Antigen presentation: The APC function of B cells through T cell interaction resulting in somatic hypermutation, B cell expansion and development of memory B cells and antibody producing B cells. B) B cells produce cytokines to mediate an immune response and influence the effector functions of immune cells. C) B cells produce antibodies which can regulate the innate and adaptive immune response. Key: maroon double Y: B cell receptor, red oval: pathogen, purple circles: cytokines, green Y, pink double Y and blue snowflake: Immunoglobulins. Image adapted from (Chan et al., 2014).*

The B cell receptor (BCR) like the TCR consists of a transmembrane and cytoplasmic domain. Intracellular signalling is transmitted by two accessory proteins Igα and Igβ which form a heterodimer and contains the ITAMs (Murphy et al., 2012). The B cell co-receptor complex consisting of CD19, CD21 (CR2) and CD81 (TAPA-1) provides additional activation signals through binding of CD21 to C3d-tagged antigens on the immunoglobulin. The cross-link between the co-receptors and the BCR induces phosphorylation of tyrosine kinase on the cytoplasmic tail of CD19 initiating a secondary signalling pathway through the phosphorylation of PI 3-kinases. The interaction between BCR and the co-receptors serves to strengthen intracellular signalling upon antigen recognition. B cells furthermore undergo T cell-dependent activation whereby a second activation signal is generated when the CD40 molecule on the B cells interacts with the CD40 ligand (CD40L) on activated T cells. Upon activation, B cells undergo clonal expansion and differentiate into memory B cells and antibody producing plasma cells and secrete cytokines (Figure 1.3). B cell cytokine responses are known to play an important role in responses against TB (du Plessis et al., 2016).

As stipulated above, the function of B cells has been defined in terms of its role when the immune system encounters a foreign invasion, but this mechanism occurs in conglomerate with other immune responses because B cells cannot eradicate *Mtb* from the host in isolation. During TB, B cells accumulate in follicle-like aggregates at the periphery of the granuloma while some penetrate the granuloma structure where they influence immune responses against *Mtb* (Ulrichs et al., 2005, 2004). In the absence of B cells, their APC function is altered in the granuloma, there is a decrease in the differentiation and frequency of *Mtb* specific T cells in the granuloma (Phuah et al., 2016). B cells can modulate immune responses in the granuloma during *Mtb* infection. The decreased frequency of circulating B cells in the peripheral of TB patients could possibly account for the dissociation in the granuloma. Patients with TB have a lower frequency of B cells and their frequency and proliferation ability is restored after TB treatment (Joosten et al., 2016).

3.2.1 Memory B cells

Memory B cells are generated in the germinal center in secondary lymphoid tissues during a primary immune response. Memory B cells have unique features to that of

naïve B cells which include; a longer lifespan, rapid and enhanced secondary immune responses upon a re-encounter with a specific antigen and robust clonal expansion. In TB patients, memory B cell frequencies are decreased and their role in the adaptive immune response is downregulated (Joosten et al., 2016). However, the frequency of plasma B cells which differentiated from B memory cells remain unchanged in patients infected with *Mtb* and in patients with TB (Joosten et al., 2016). A similar trend was observed in obese T2D patients where the frequency of resting memory B cells decreased in comparison to healthy individuals while the plasma cell frequencies remained unchanged ($CD19^{+}CD27^{+}$)

3.2.2 Regulatory B cells

Invading pathogens and the presence of tissue damage generally induces an inflammatory response to clear the pathogen or to repair any occurrence of tissue damage. However, if this inflammation remains constant and unchecked it can be pathogenic to the host. The immune system counteracts this through the release of anti-inflammatory cytokines and the cells from which they are secreted are called immunosuppressive cells which have immune-regulatory function (Medzhitov, 2008).

During inflammation, there are defects in the circulation of these suppressor cells (Vered et al., 2013). The B cell subset associated with this immunosuppressive function are known as regulatory B cells (Bregs). B cells account for 2-10% of all immune cells in human whole blood and an even lower frequency are Bregs (<1%) (Iwata et al., 2011). Bregs originate from transitional 2 marginal zone precursor B cells, these are immature B cells occurring during B cell developmental stage. After B cell maturation, B cells migrate from the bone marrow to the spleen where they differentiate into transitional type 1 or type 2 B cells.

The phenotype of Bregs is characterized by the secretion of anti-inflammatory cytokine IL-10 and the production of IgM (Mauri and Bosma, 2012). Bregs have been primarily studied in autoimmune diseases where their frequency is higher in comparison to the absence of autoimmunity (Blair et al., 2010; Correale et al., 2008). IL-10 dependent $CD19^{+}CD24^{hi}CD38^{hi}$ Breg phenotype suppresses Th1 responses and Th17 differentiation (Blair et al., 2010; Flores-Borja et al., 2013). The suppressive ability of Bregs is expressed through the secretion of IL-10 and transforming growth factor-β (TGF-β) and their engagement with co-stimulatory molecules (CD80/CD86).

$CD19^{+}CD24^{hi}CD38^{hi}$ Breg expressing $CD1d^{hi}$ and IgD inhibit the production of IFN-γ and TNF-α through $CD4^{+}$T cell activation. (Lemoine et al., 2011). Bregs control the production of excessive pro-inflammatory cytokines through inhibiting the differentiation of $CD4^{+}$T cells into Th1 and Th17 (Flores-Borja et al., 2013). The continuous exposure of Bregs to pro-inflammatory cytokines IL-1β and IL-6 impair their development and suppressive ability (Flores-Borja et al., 2013).

An alternative mechanism for B cells to regulate their immunosuppressive properties is through the expression of the death-inducing ligand FasL (CD178). FasL is a membrane bound protein belonging to the TNF-family and the Fas/FasL pathway mediates lymphocyte apoptosis (Zuñiga et al., 2002). B cells expressing Fas on their cell surface bind to target cells expressing Fas, activating the Fas/FasL cascade induce apoptosis of the target cell. (Lundy et al., 2001).

3.2.3 Regulatory B cells in TB

The regulatory function of B cells can also be induced by IL-5, the surface expression of IL-5 receptor alpha (IL-5rα) is higher in $FasL^{+}$ expressing B cells in comparison to $FasL^{-}$ B cells (Klinker et al., 2013). The expression of both $FasL^{+}$ IL-5rα in Bregs in TB patients increases with TB treatment. This could be suggestive of a protective role that the expression of FasL has because B cells expressing FasL can induce apoptosis and probably induce the apoptosis of *Mtb* (van Rensburg et al., 2017). A similar trend was observed in killer Bregs expressing the phenotype $CD19^{+}CD38^{+}IgM^{+}FasL^{+}$IL5Rα (van Rensburg et al., 2017).

3.3 B cells in T2D

In patients with T2D the depletion of B cells was correlated with a decline in Th17 proliferation and an increased production of pro-inflammatory cytokines (Cao et al., 2016). Furthermore, B cells are implemented in the progression of insulin resistance in T2D through the activation of Th1 and Th17 cells and the release of IgG an antibody elevated during the presence of a pathogen within the host (DeFuria et al., 2013). The secretion of Th2 cells suppress the inflammatory effects of Th1 cells through stimulating the differentiation of B cells into plasma cells and memory B cells to enhance an immune response (Cao et al., 2016). The frequency of circulating naïve B cells is higher while memory B cells were lower in patients with T2D when compared to non-diabetics (Nam et al., 2018).

The mechanism and effectiveness of B cells is impaired in TB and this is evident through the reduction of naïve and memory B cells, the secretion of pro-inflammatory cytokines and the decrease of immunosuppressive Bregs mediated by IL-10. In T2D the defects in B cells has been associated with obesity and insulin resistance with limited research done to examine the effects of hyperglycaemia on these cells. Little is known about the role of B cells in TB patients with T2D (TB-T2D). Preliminary data generated in our research group suggest that the absolute number of B cells in TB-T2D patients are altered in comparison to TB patients without T2D (unpublished). To our knowledge the only published work on B cells in TB-T2D was conducted by Kumar et al where the frequency of naïve B cells was decreased. B cells are important contributors of the adaptive immune response, yet little is known about their function in TB-T2D patients.

Aims and Objectives

Hypothesis

We hypothesize that the frequency of B and T cell subsets as well as peripheral cytokine responses will be altered in TB patients with and without T2D.

Aim

The aim of this study was to investigate the phenotypic profile of B and T cell subsets in TB patients with and without T2D and their peripheral cytokine responses.

Objectives

1. To phenotype B and T cells isolated from the whole blood of TB patients with and without T2D through cell surface staining of cells collected at baseline (prior to initiation of TB treatment) and month two of TB treatment.

2. To investigate peripheral cytokine concentrations of TB patients with and without T2D at baseline and month two of TB treatment.

Chapter 2

1. Ethics statement

This research study was conducted in accordance with the ethical principles for research established by the Declaration of Helsinki, the International Conference of Harmonization (ICH) and Good Clinical Practice (GCP) guidelines.

Ethical approval for the ALERT (N13/05/O64A), TANDEM (N13/05/064) and Screen TB (N16/05/070) studies were obtained from the Health Research Ethics Committee (HREC) of Stellenbosch University as well as the City of Cape Town City Health. Informed consent specifying clinical and research laboratory procedures were read and explained by study coordinators and signed by participants.

2. Participate recruitment

2.1 Study participants

For this cross-sectional study, participants were recruited from clinics surrounding Tygerberg Hospital (Fisantekraal, Ravensmead, Uitsig, Adriaanse and Sarepta) in the Western Cape. Recruited participants were enrolled into the ALERT, TANDEM and Screen TB studies if they met the study specific inclusion criteria. Close contacts (CCs) of TB patients with and without T2D, were enrolled in the ALERT study. Healthy controls and TB patients, both with and without T2D were enrolled in the TANDEM study. As part of this study TB patients (TB and TB-T2D) were followed up during TB treatment. For the Screen TB study, participants that presented with symptoms suggestive of TB were recruited and followed up during TB treatment. Participants who presented with symptoms suggestive of TB including, cough, fever, haemoptysis, chest pains, night sweats, loss of appetite, weight loss, malaise were included in the study.

Participants were between the age of 18 and 65 were excluded if they were HIV positive, pregnant or breastfeeding, had a haemoglobin less than 9g/L, were currently on TB treatment or received TB treatment 90 days prior to recruitment, used antibiotics such as quinolone or aminoglicozide or immunosuppressive medication including

corticosteroids 60 days prior to recruitment or presented with infectious disease other than TB and ulcerations were excluded. T2D was diagnosed as haemoglobin A1c (HbA1c) ≥ 6.5%.

2.2 Selection of participants

Serum samples of 59 participants were included for luminex analysis (Section 3). Thirty-four were TB cases and five were TB-T2D patients, twenty-five of the TB cases and two of the TB-T2D patients had a month two follow up. Eleven T2D patients (4 QFN positive and 7 QFN negative) and nine healthy controls (HC) (all QFN positive) were also included (Figure 2.1). Cryopreserved whole blood of 54 participants were selected for the phenotypic analysis of B cells (Section 4). Of the 54 participants, 15 were TB cases and three TB-T2D patients, 11 of the TB patients were followed up two months into anti-TB treatment and those samples were included (Figure 2.1). Twenty T2D patients (14 QFN positive and 6 QFN negative) and six healthy controls (HC) (all were QFN positive) were included (Figure 2.1).

2.3 Clinical Tests

Pulmonary TB was diagnosed in TB patients using the mycobacterial growth indicator tube (MGIT) (Becton Dickinson (BD), Borstel, Germany) and GeneXpert (Cepheid, California (United States America (USA))) tests. Latent TB infection (LTBI) was confirmed in HCs using the QuantiFERON (QFT)-TB Gold Plus test (Qiagen, Venlo, Netherlands).

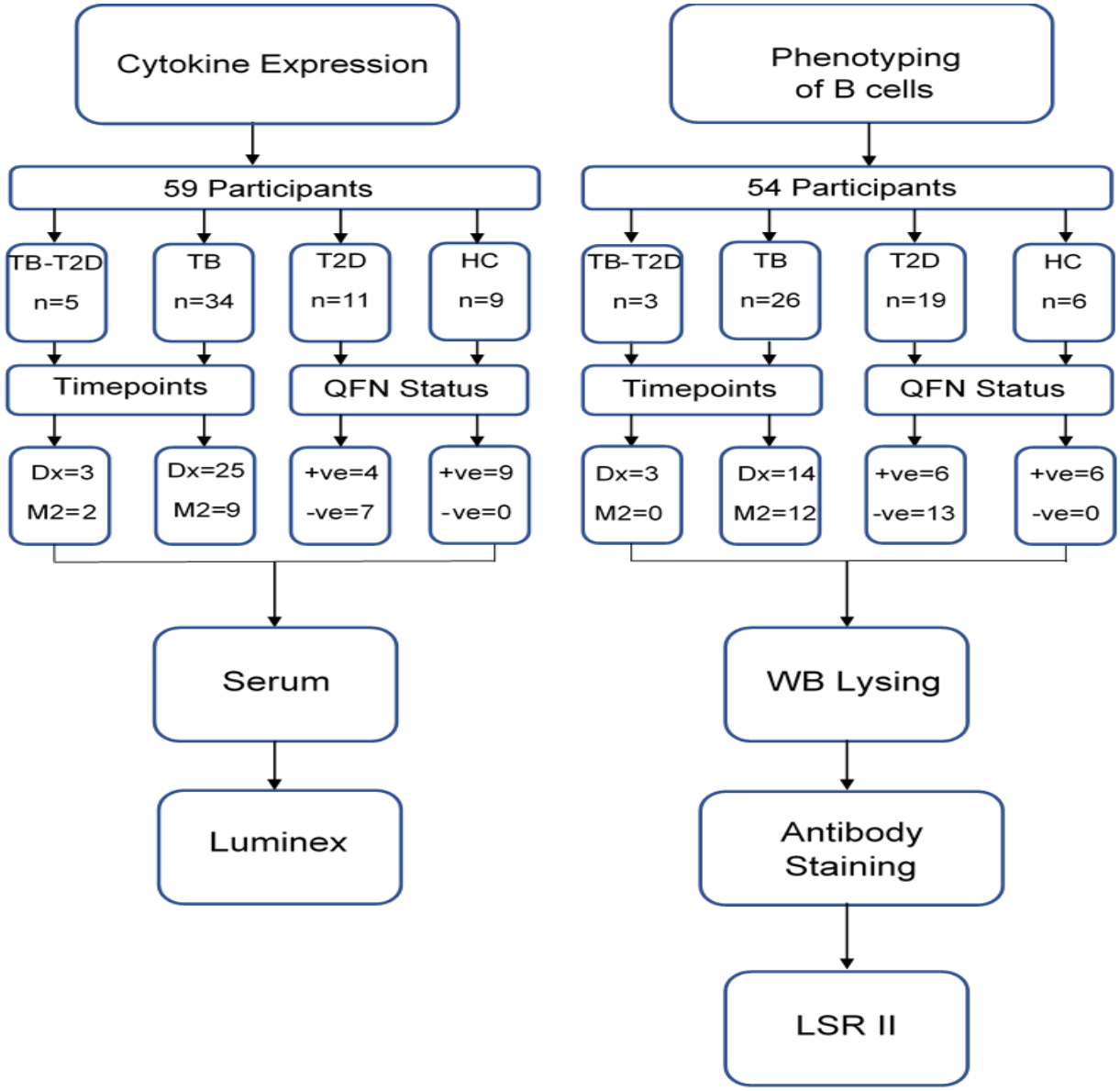

Figure 2.1: Classification of study participants and the downstream analyses performed. Study participants were classified as follows: HCs, T2D, TB and TB-T2D. Two time-points, diagnosis (Dx) and month 2 (M2) into anti-TB treatment, was included for TB and TB-T2D patients. The QuantiFERON status of T2D patients and HCs were noted for as negative (QFN -ve) and positive (QFN +ve).

2.3.1 QuantiFERON test

One ml of whole blood collected in Lithium Heparin (LiHep) tubes (Becton Dickinson (BD), Borstel, Germany) was transferred into QuantiFERON-TB Gold Plus tubes namely Nil, TB1, TB2 and mitogen tubes (Qiagen). The tubes were incubated at 37ºC in a CO_2 incubator for 20 hours. After 20 hours the tubes were centrifuged at 3000 x g for 15 minutes. Supernatant was collected and transferred to cryotubes and stored at -80ºC until the QuantiFERON ELISA assay was performed.

The QuantiFERON-TB Gold Plus ELISA (Qiagen) was done in accordance to the manufacturer's instructions. Briefly, samples were thawed and pulse vortexed. The

samples, standards and internal controls were run in duplicate. The plate was covered with foil and incubated for two hours at RT. The wells were washed six times with wash buffer using an automated plate washer (Bio-Rad, California (USA)). Enzyme substrate solution was added into each of the wells and the plate was covered with foil and incubated for 30 minutes at RT. After incubation, enzyme stopping solution was added into each of the wells and the plate was read after five minutes of adding the stop solution. The plate was read on the iMark Microplate reader (Bio-rad) using the QuantiFERON-TB Gold analysis software version 2.71.

2.3.2 MGIT test

A sputum sample was collected from all study participants and processed in the Biosafety level 3 (BSL3) laboratory. One ml of sputum was transferred to a 50ml tube and one ml of MycoPrep (Sigma-Aldrich, St. Louis, USA) added to the sample. It was then vortexed for 15 seconds and incubated at room temperature (RT) for 15 minutes. During incubation the samples was vortexed for five second every five minutes. Thereafter a phosphate (PO_4) buffer was added up to the 25ml mark and the tube inverted until the content was thoroughly mixed. The sample was then centrifuged at 3000 x g at 16ºC for 15 minutes, the supernatant discarded and the pellet resuspended in one ml of PO_4 buffer.

A lysophilized vial of BBL MGIT PANTA (Sigma-Aldrich, St. Louis, USA) antimicrobial agent mixture was reconstituted with 15ml of BACTEC MGIT growth supplement and 800µl of the PANTA and 500µl of decontaminated sputum added to the MGIT tube. MGIT tubes were then placed in a MGIT 960 instrument (BD) until the samples flagged positive or for 42 days if negative. Time to positivity (TTP) was recorded for each sample and a TTP of less than four days was noted as contaminated and these participants were excluded from the study.

2.3.3 GeneXpert

One ml of sputum and two ml of Xpert MTB/RIF Sample Reagent (SR) (Cepheid, California (CA) (USA)) was added into a tube and incubated for 10 minutes at RT. The sample was then transferred to a GeneXpert MTB/RIF cartridge ensuring the lid was tightly sealed. The cartridge was then transferred to the GeneXpert Dx instrument (Cepheid, CA, (USA)) and tested using the GeneXpert software (GeneXpert Dx system version 4.7b).

3. Cytokine expression

3.1 Sample preparation

Whole blood of study participants was collected in nine ml serum tubes (BD, Germany). Blood samples were centrifuged at 2 000 x g for 10 minutes and the serum was collected and stored in 500µl aliquots at -80°C until cytokine analysis using the luminex platform (Section 3.2).

3.2 Luminex multiplex immunoassay

The concentrations of 41 host immune markers, which included; 1) chemokine (C-X-C motif) ligand 9 (CXCL9/MIG), Fas and matrix metalloproteinase (MMP-9) (Cat#: LXSAHM-03, R&D Systems, Minneapolis, USA); 2) Apo-AI and Apo-CIII (Cat#: APOMAG-62K, Merck Millipore, USA); 3) C-reactive protein (CRP) and α1-antitrypsin (Cat#: HNDG2MAG-36K, Merck Millipore, USA); 4) complement C2, complement C4b, complement C5 and complement C5a (Cat#: HCMPMAG-19K, Merck Millipore, USA); 5) soluble interleukin (sIL) 2 receptor alpha (sIL-2Rα), sIL-4 receptor (sIL-4R) and sIL-6 receptor (sIL-6R) (Cat#: HSCRMAG-32K, Merck Millipore, USA); 6) eotaxin, granulocyte macrophage-colony stimulating factor (GM-CSF), G-CSF, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-Ira, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, soluble interferon gamma inducible protein 10 (IP-10), macrophage-derived chemokine (MDC), macrophage inflammatory proteins (MIP-1α), MIP-1β, transforming growth factor-α (TGF-α), TNF-α, TNF-β, vascular endothelial growth factor (VEGF) (Cat#: HCYTOMAG-60K, Merck Millipore, USA) were measured in the serum samples (using the kits indicate in brackets) of the participants described above (Figure 2.1).

The assays were conducted in accordance to the manufacturer's instructions. Briefly, the beads were sonicated for 30 seconds and then vortexed for 1 minute before they were mixed. After adding all the beads to the mixing bottle, a set volume was added to each well. The plates were incubated for 2 hours at RT and some plates were incubated overnight on the shaker at 4°C. Detection antibody was then added into each of the wells and incubated for an hour on the shaker at RT. Streptavidin phycoerythrin was added at the end of incubation and placed on the shaker to incubate for 30 minutes on the shaker at RT. Washes were done on the plates on each of the wells at the end of every incubation. On the last wash, sheath fluid was added to each of the wells on the plates. Plates were read using the Bio-Plex platform (Bio-Plex™,

Bio-Rad Laboratories, Hercules, CA, USA) (supplier) and the concentration of each analyte determined using the Bio-Plex manager software version 6.1 (Bio-Rad Laboratories).

The serum samples of kits 1-5 were diluted with serum matrix in a; 1:2, 1:4000, 1:2000, 1:200 and 1:5 respectively and the samples in kit 6 were ran neat. All the samples, quality and internal controls were evaluated in duplicates. The quality and internal control values passed for all the kits as they were within the recommended range as stipulated by the manufacturer.

4. Phenotyping of B cells

4.1 Whole blood lysing

Red blood cells (RBCs) of one ml of whole blood (WB) collected in Sodium Heparin (NaHep) tubes (BD, Germany) were lysed by adding nine ml of fluorescence activated cell sorting (FACS) lysing solution containing a fixative (1:10 dilution) (BD, (USA). After lysing the RBCs, the cells were washed twice with phosphate buffered saline (PBS) (Thermo Fisher Scientific, Massachusetts, United Kingdom (UK)) and centrifuged at 400 x g for 10 minutes. Thereafter, the cells were cryopreserved in one ml cryo medium consisting of 90% fetal bovine serum (FBS) (Biowest, Missouri, USA) and 10% Dimethyl sulfoxide (DMSO) (Millipore, Billerica, MA, USA). The pellets were stored overnight at -80°C in Mr frosty containers containing isopropanol (Fisher Scientific, Loughborough, UK) and then transferred and stored at -80°C freezer.

4.2 Thawing of Whole Blood samples

Cells were retrieved from the -80°C freezer and placed in a 37°C water bath. Before the pellets were completely thawed, the cells were transferred in a dropwise manner into a 15ml tube containing 10ml of PBS. The cells were centrifuged at 400 x g for 10 minutes, and the pellet washed with 10ml PBS, after which the cells were stained as described in section 4.4.

4.3 Cell Stimulation

For optimization experiments, cells were stimulated with phytohemagglutinin (PHA) (Bioweb, Johannesburg, South Africa). After thawing and washing of cells, cells were transferred to 96 well round bottom plates (Thermo Fisher Scientific, UK) and 5µg/ml of PHA was added to each well. The cells were stimulated for 16 hours inside a 37ºC

incubator containing CO_2. At the end of the stimulation, the cells were washed twice with PBS and centrifuged at 400 x g for 10 minutes in between wash steps.

4.4 Cell Surface Staining of Whole Blood

PHA stimulated cells were resuspended in FACS buffer (2% FBS in PBS), transferred into FACS tubes and centrifuged at 400 x g for five minutes. Cells were resuspended in 20µl of FACS buffer (unstained cells) or in 20µl of antibody mix containing the concentration of antibodies as determined in the titration experiment (section 4.5). Cells were incubated at 4ºC (in the dark) for 45 minutes. Cells were washed twice with 200µl FACS buffer and centrifuged at 400 x g for five minutes. After the last wash step, the pellet was resuspended in 200µl FACS buffer and stored at 4ºC until acquisition with the 13-parameter LSR II instrument from BD equipped with BD FACS Diva software v8.0.1 (BD, Germany). On average, 400 000 events were acquired for each sample. Data obtained from the LSR II was analysed using FlowJo Version 10 software (Treestar, San Carlos, CA)

4.5 *Titration experiment*

Prior to staining the study participant samples, antibody titrations were conducted to determine the optimal antibody concentration where non-specific binding of antibodies are minimal and a clear separation between the negative and positive cell populations are detected. The following markers were included in the WB FACS panel and therefore titrated: CD3 (T cell marker) (PerCP-Cy5.5), CD4 (T helper cell marker) (BV421), CD8 (Cytotoxic T cell marker) (APC-R700), CD5 (B-1a cell marker) (BV510), CD19 (B cell marker) (FITC), CD125/IL-5 receptor alpha (IL-5Rα) (PE), CD178 (FasL) (APC), IgM (PE-Cy5), CD24 (B cell activation marker) (APC-H7), CD27 (Memory B cell marker) (PE-CF594) and CD38 (activation marker) (BV605). All the antibodies were purchased from BD (BD, Germany). Each antibody was titrated with the starting concentration being 2-fold higher than the manufacturer's recommended concentration. A 2-fold serial dilution was prepared using FACS buffer (Figure 2.2).

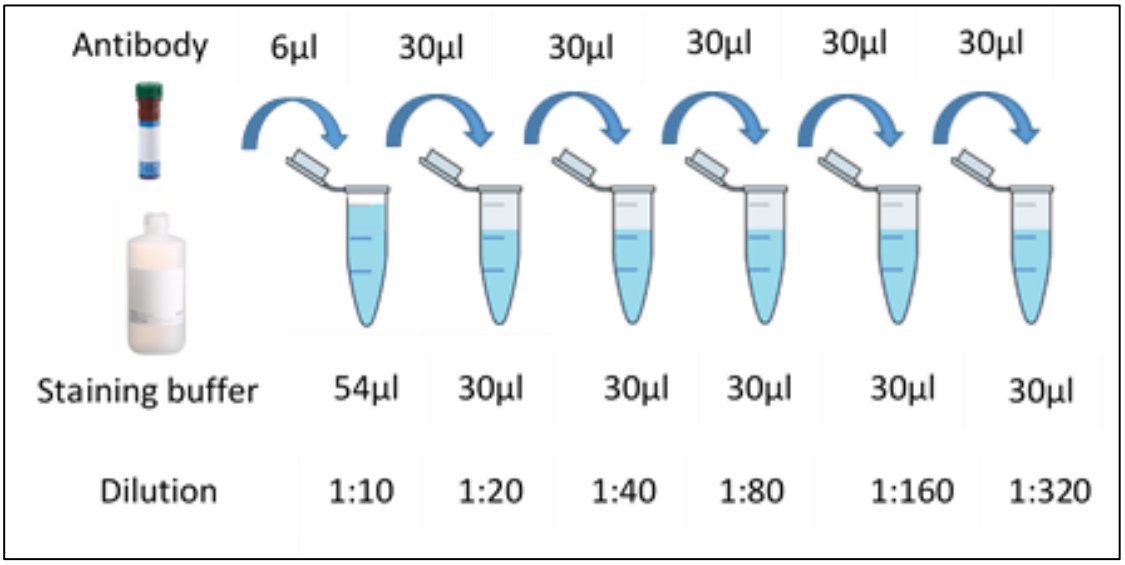

Figure 2. 2: Serial dilution used for FACS antibody titration experiments. The starting dilution in the dilution series (1:10) was 2-fold higher than the manufacturers recommended concentration for each antibody (1:20). From the 1:10 dilution the antibody was further diluted (1:2) by adding 30µl of the 1:10 dilution to a tube containing 30µl of staining buffer (2%FBS in PBS). Thereafter, 30µl each tube was transferred to the next containing 30µl of staining buffer. This process was repeated for a total of five concentrations.

During titration experiments, an unstained sample serving as a negative control was used to determine the position of the negative gate that should be applied to all samples. This gate was then used determine the optimal concentration for the antibody (Figure 2.3). The median fluorescent intensity (MFI) from each dilution of the antibody was obtained and the signal to background noise ratio calculated. The concentration with the highest signal to noise ratio for each antibody was selected as the optimal concentration as indicated in Figure 2.3 and 2.4.

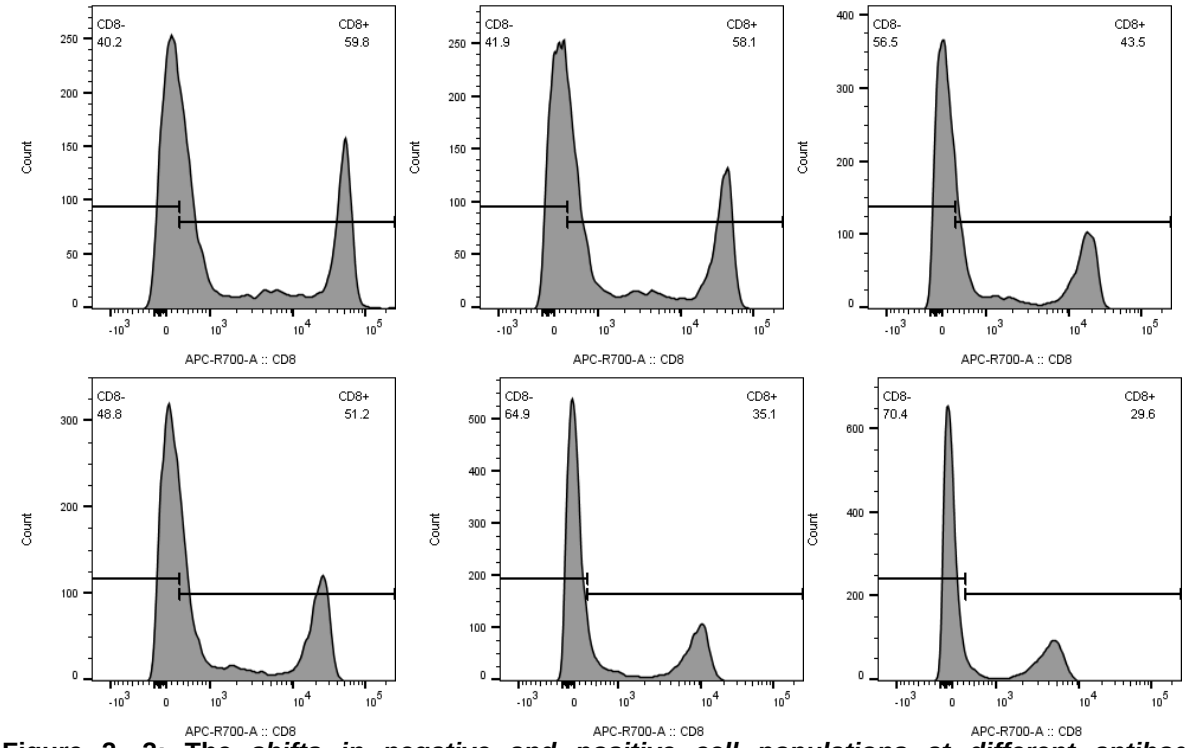

Figure 2. 3: The *shifts in negative and positive cell populations at different antibody concentrations included in the serial dilution.* *The titration of CD8 (APC-R700) was used to illustrate the shift in negative and positive cell populations. A 2-fold dilution series was done starting from a dilution of 1:10 to 1:320 (from top left to bottom right). As illustrated above, the lower concentrations showed a clear distinction between the cell populations as observed. The histograms were obtained from FlowJo version 10.*

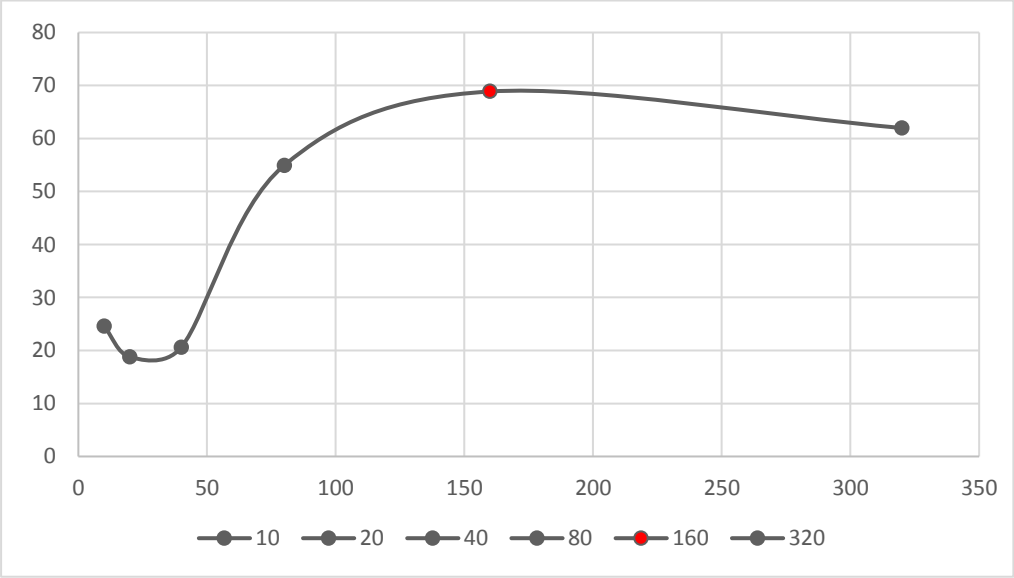

Figure 2. 4: Dilutions vs ratio to background noise plot. The MFIs of the negative and positive CD8 cell population were obtained from FlowJo version 10. The background noise to signal ratio was measured by dividing the positive median by the negative median of the same concentration for each of the dilutions. In this example, the highest fluorescent intensity was detected for the 1:160 dilution, noted in red on the graph. This concentration was used in subsequent experiments.

4.6 Antibody compensation

The fluorophores conjugated to antibodies can be excited by more than one laser and the detectors can pick up signals from different fluorophore resulting in a spill over. Therefore, the aim of a compensation was to detect whether there was fluorophore spill over in the different detection channels. Compensation was done using compensation beads. One hundred µl of FACS buffer was added into FACS tubes followed by one drop of negative and positive compensation beads (BD Biosciences). Antibodies were added to the relevant FACS tubes as per the selected MFI concentration and an unstained with positive and negative beads was used as control. The beads were pulse vortexed and incubated at 4ºC (in the dark) for 45 minutes. The beads were washed with two ml of FACS buffer and centrifuged at 200 x g for 10 minutes. Beads were resuspended in 500µl FACS buffer and stored at 4°C (in the dark) until acquisition. Post-acquisition on the LSR II, the compensation was calculated, and a correction performed to correct for the overspill in each channel.

4.7 Fluorescence Minus One (FMO) Experiment

Our flow panel had eleven colours and as explained in the previous section this would increase the chances of background staining as a result of fluorescent spill over, resulting in false positive signals. The aim of an FMO experiment was to identify whether fluorophores spilt over into other channels and to resolve problems that may have not been easily detected during compensation. FMO controls are important when the panel measures markers with rare events as was evident in this panel. Lysed WB was added into 12 FACS tubes, all but one antibody was added to each tube with a different antibody excluded in each of the tubes. The staining protocol as stipulated in section 4.4 was used. Samples were acquired using the LSR II instrument and the data analysed using FlowJo. WB samples stimulated with PHA (5µg/ml) were stained and tested prior to thawing the WB samples obtained from the study participants themselves.

4.8 Gating Strategy

Samples were run in batches of 20 and an unstained sample was included for each study participant. The following gating strategies were used: a time gate was done to assess the quality of the run and to test if there were any disturbances during sample acquisition. Doublets were excluded through gating for single cells based on forward scatter height (FSC-H) and FSC area (FSC-A). Lymphocyte populations were selected based on cell size and granularity of the cells by gating on the side scatter area (SSC-A) and FSC-A. Table 2.1 illustrates the B and T cell phenotypes that were assessed for this study.

Table 2.1: The phenotypes and their characterization.

Phenotype	Characterization
$CD4^{+}CD38^{+}$	Activated T-helper cells
$CD8^{+}CD38^{+}$	Activated cytotoxic cells
$CD19^{+}CD27^{+}$	Resting memory B cells
$CD19^{+}CD24^{+}CD27^{+}$	Activated memory B cells
$CD19^{+}CD5^{+}IgM^{+}CD38^{+}CD125^{+}CD178^{+}$	Killer B cells
$CD19^{+}CD5^{+}IgM^{+}CD38^{+}CD125^{-}CD178^{+}$	Killer B cells
$CD19^{+}CD5^{+}CD24^{+}CD38^{+}$	Bregs
$CD19^{+}CD24^{hi}CD38^{hi}$	Bregs
$CD19^{+}CD24^{hi}CD38^{hi}CD125^{+}CD178^{+}$	Killer Bregs
$CD19^{+}CD24^{hi}CD38^{hi}CD125^{-}CD178^{+}$	Killer Bregs

To characterize the expression of T cells, the lymphocyte gate was used to plot for CD3 positive cells. T helper (CD4) and cytotoxic (CD8) T cells were plotted from CD3+ cells and their activated function was determined through gating for CD4+ and CD8+ cells that expressed CD38 respectively (Figure 2.5.1).

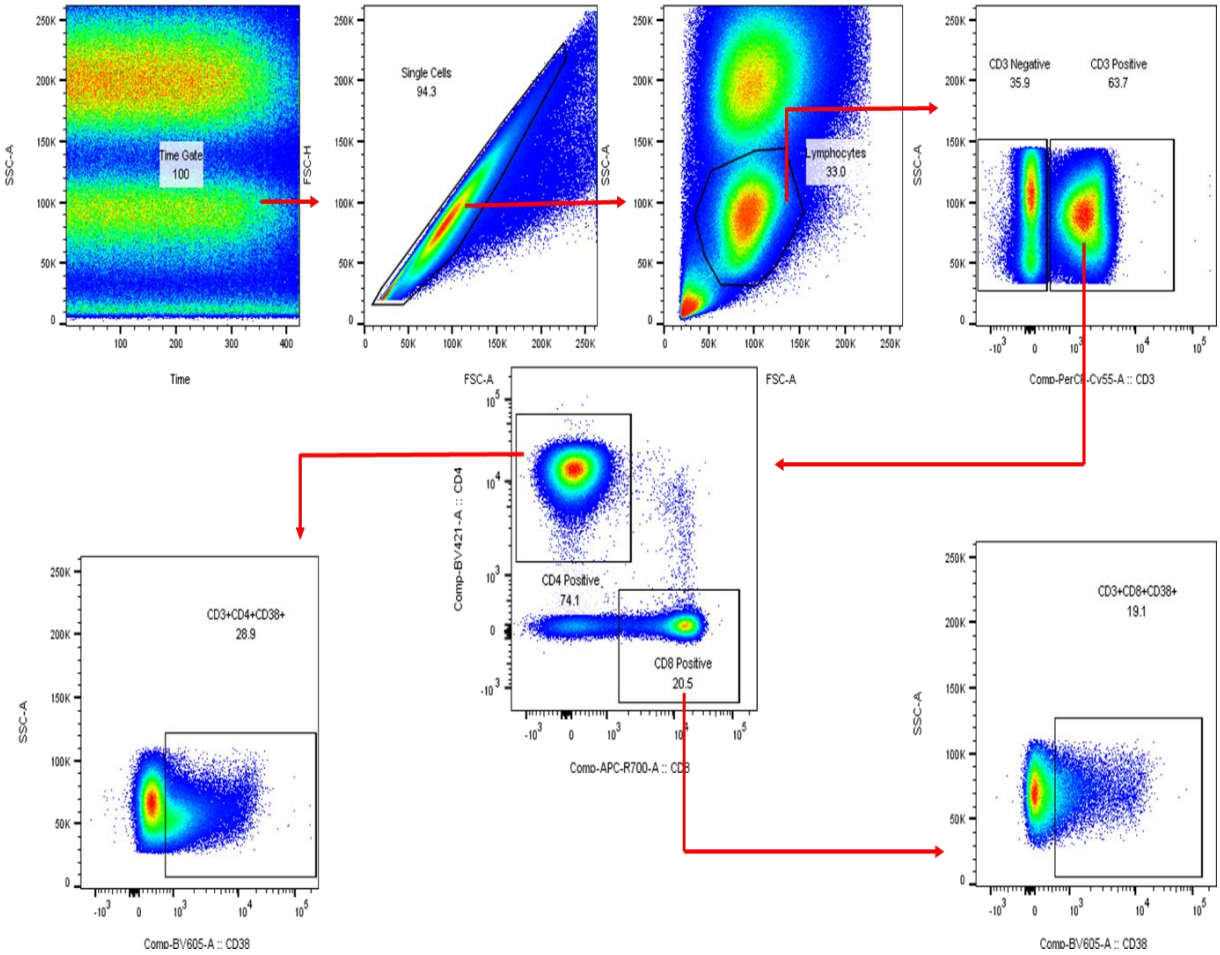

Figure 2.5.1: The gating strategy for the characterization of activated T cells. Shown above are flow cytometry plots retrieved from an analysis done on FlowJo version 10. From left to right the time gate, single cells and lymphocytes were selected based light scatter signal. $CD3^+$ cells (T cells) were selected and gated for $CD4^+$ (T-helper cells) and $CD8^+$ (Cytotoxic cells) and their activation function characterized through their expression of $CD38^+$ marker.

B cells were determined through gating on the CD3 negative cells then $CD19^+$ for the identification of B cells and their apoptotic ability was investigated through the expression of the following phenotypes $CD125^-CD178^+$ and $CD125^+CD178^+$ (Figure 2.5.2). In Figure 2.5.3 memory B cells a subpopulation of B cells was determined through using the same gating strategy for B cell identification were $CD27^+$ cells were defined as resting memory B cells and $CD24^+CD27^+$ as activated memory B cells. Another function of B cells that was investigated was that of killer B cells which were defined through firstly gating on $CD19^+IgM^+$ followed by gating on $CD5^+CD38^+$ and killer function was determined through gating on $CD125^-CD178^+$ and $CD125^+CD178^+$ as shown on Figure 2.5.4.

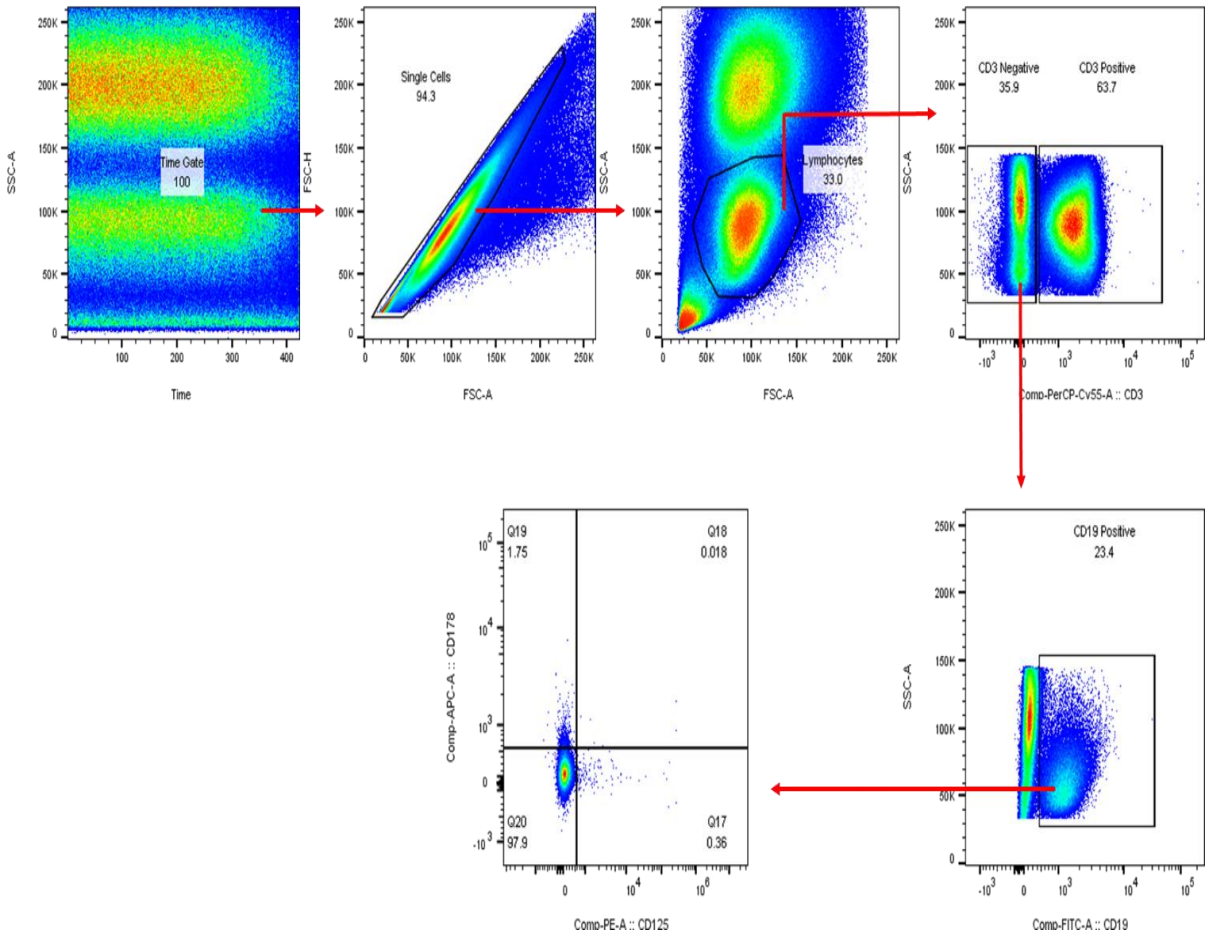

Figure 2.5.2: The gating strategy of B cells. Shown above are flow cytometry plots retrieved from an analysis done on FlowJo version 10. From left to right the time gate, single cells and lymphocytes were selected based light scatter signal. The characterization of B cells was done through gating on CD3 negative cells and plotting on $CD19^+$ cells. Their killer function was accessed through the expression of $CD125^+CD178^+$ and $CD125^-CD178^+$ phenotypes.

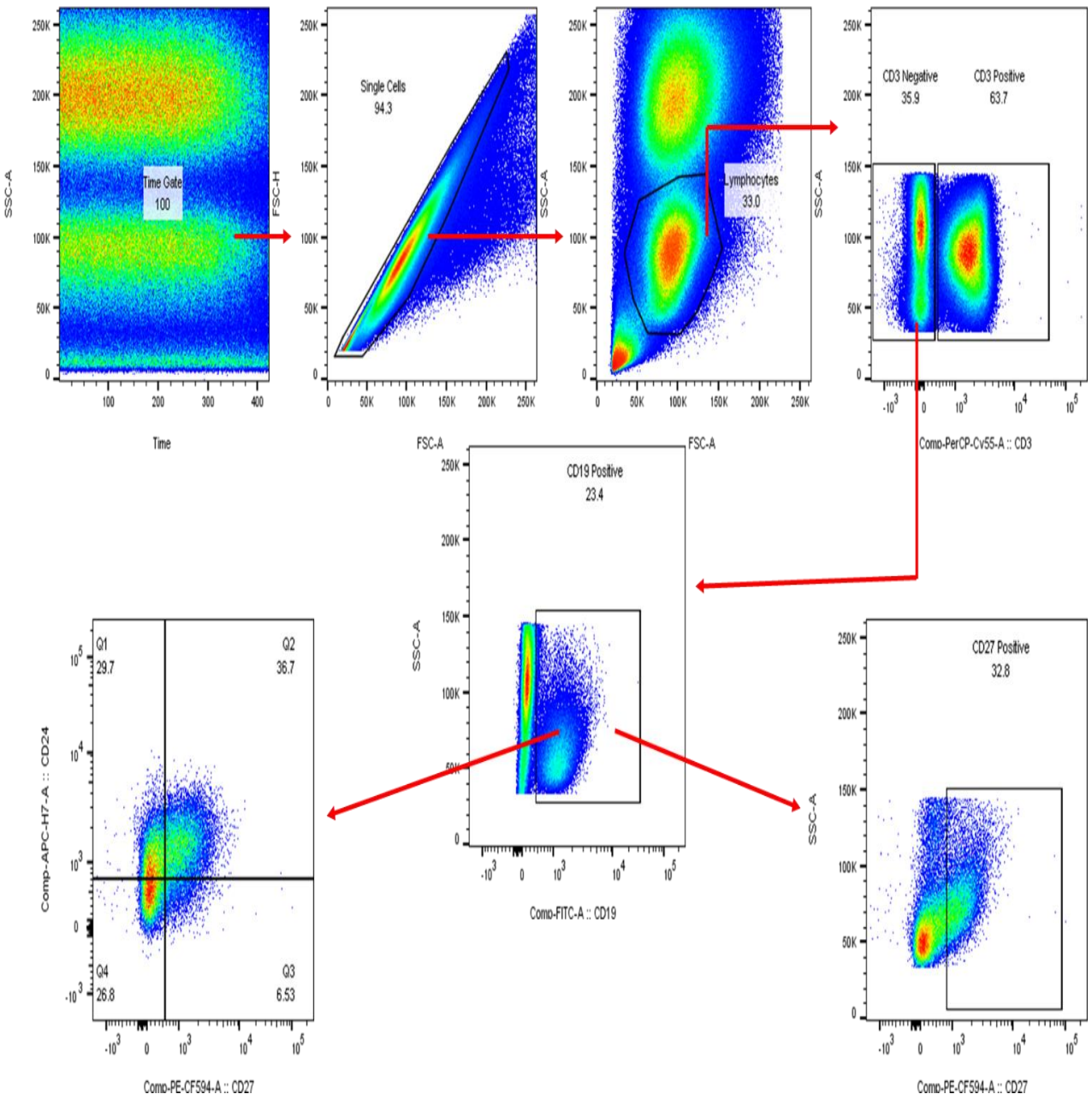

Figure 2.5.3: ***The gating strategy of Memory B cells.*** *Shown above are flow cytometry plots retrieved from an analysis done on FlowJo version 10. From left to right the time gate, single cells and lymphocytes were selected based light scatter signal. CD3⁻ cells were gated on for the characterization of CD19⁺ cells which were gated for resting memory B cells CD27⁺ and activated memory B cells CD24⁺CD27⁺.*

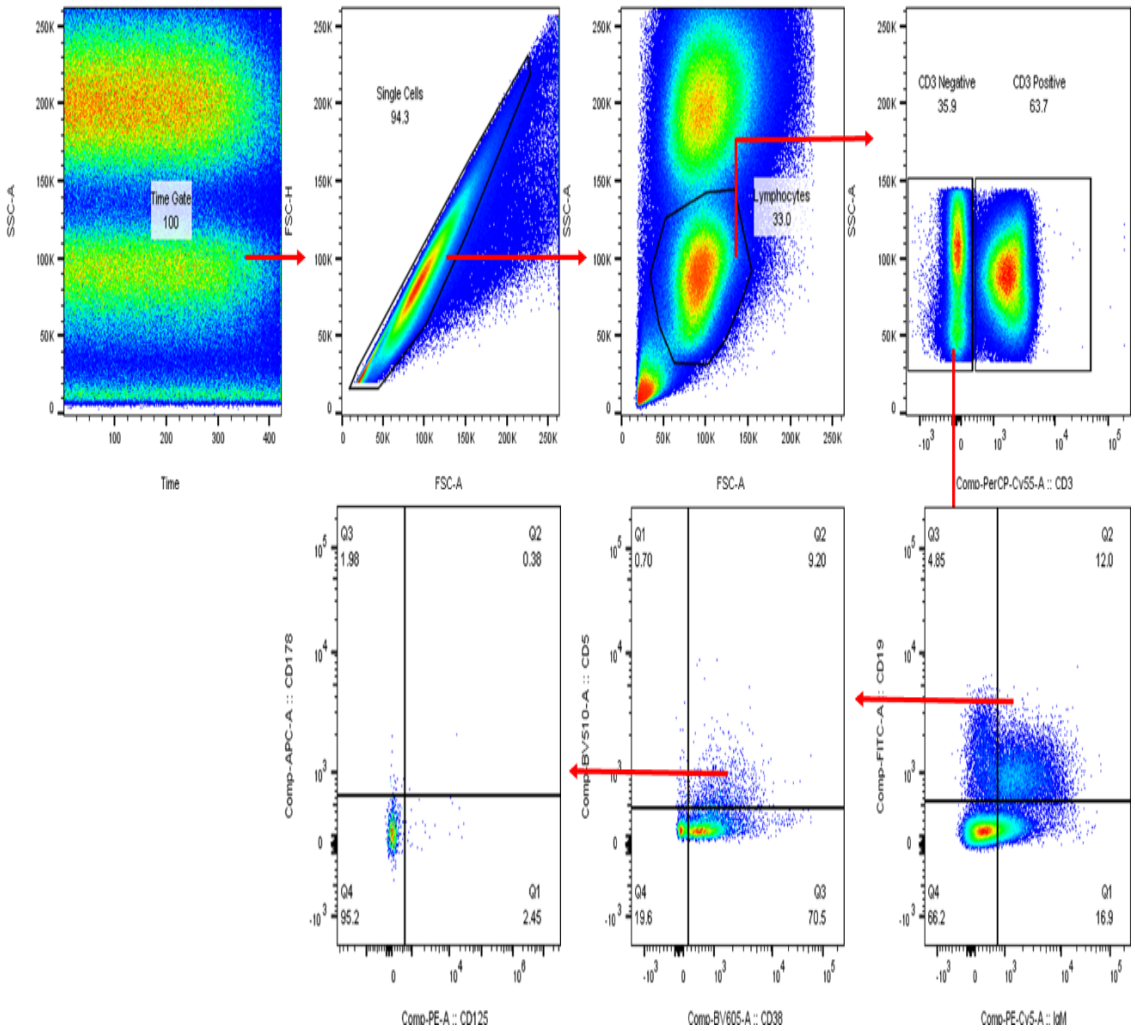

Figure 2.5.4: The gating strategy of Killer B cells. Shown above are flow cytometry plots retrieved from an analysis done on FlowJo version 10. From left to right the time gate, single cells and lymphocytes were selected based light scatter signal. $CD3^-$ cells were gated for $CD19^+IgM^+$ expression from where the expression of $CD5^+CD38^+$ plotted and their apoptotic ability was identified through the expression of $CD125^+CD178^+$ and $CD125^-CD178^+$ phenotype.

The regulatory function of B cells as defined in Table 2.1 was also investigated and the gating strategy shown in Figure 2.5.5. $CD5^+$ cells were gated from the lymphocyte population after which $CD19^+$ was gated on and $CD24^+CD38^+$ cells were defined as regulatory B cells. The final phenotype was the killer regulatory B cells (Bregs) and identification of B cells was done shown in Figure 2.5.2 after which $CD24^{hi}CD38^{hi}$ defined as Bregs and their apoptosis ability defined through the expression of phenotype $CD125^-CD178^+$ and $CD125^+CD178^+$ cells as shown in Figure 2.5.6.

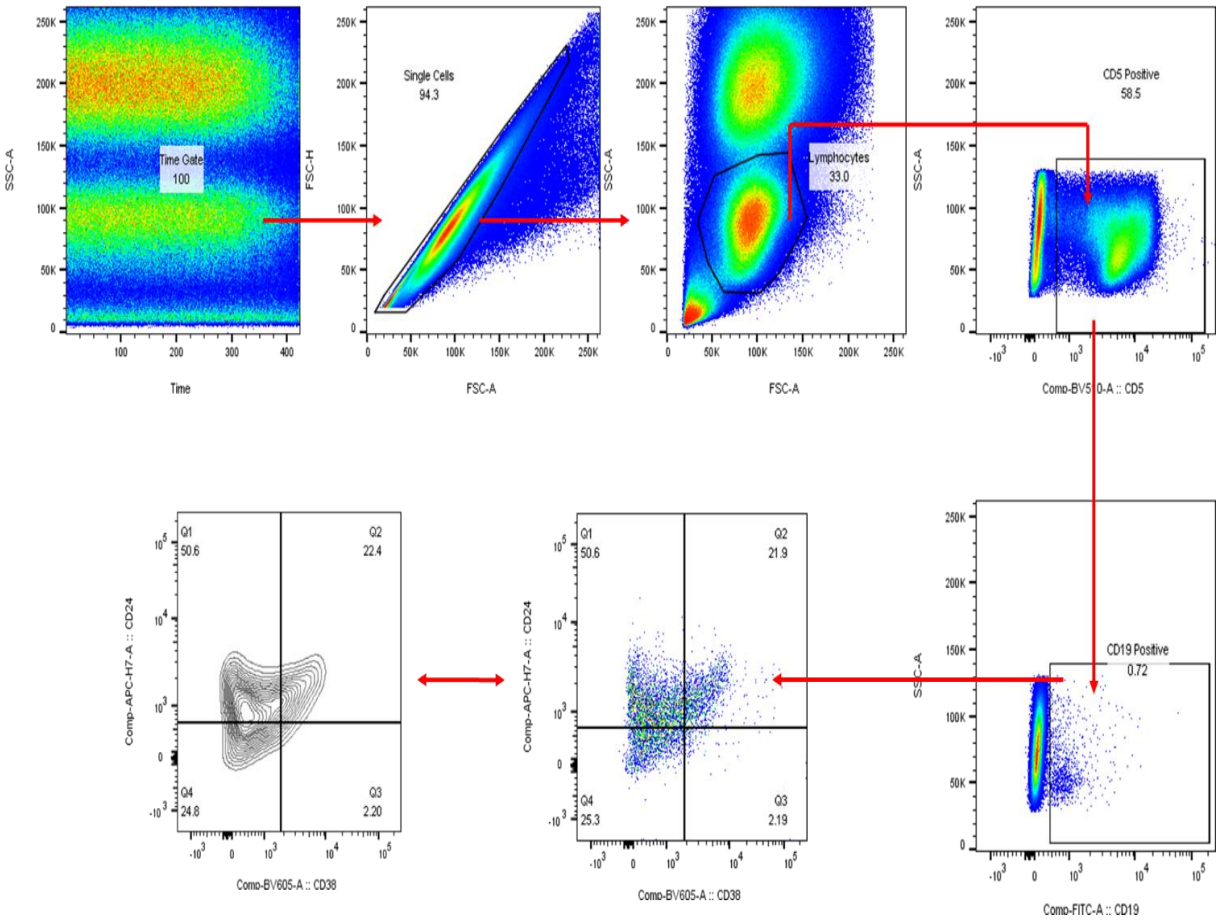

Figure 2.5.5: ***The gating strategy of regulatory B cells.*** *Shown above are flow cytometry plots retrieved from an analysis done on FlowJo version 10. From left to right the time gate, single cells and lymphocytes were selected based light scatter signal. Lymphocytes were gated for $CD5^+$ cells and $CD19^+$ was plotted to gate for regulatory B cells through the expression of $CD24^+CD38^+$ (Bregs).*

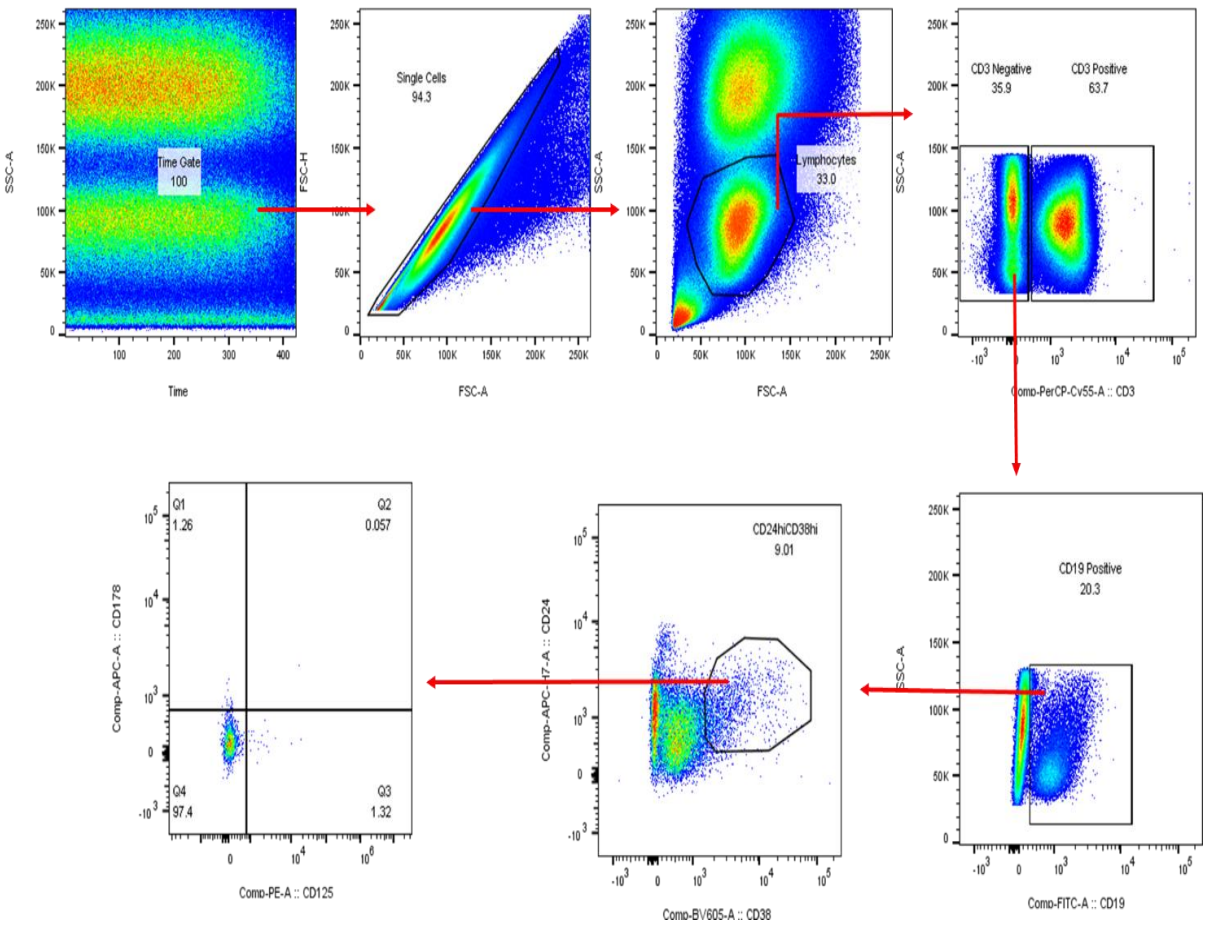

Figure 2.5.6: ***The gating strategy of killer regulatory B cells.*** *Shown above are flow cytometry plots retrieved from an analysis done on FlowJo. From left to right the time gate, single cells and lymphocytes were selected based light scatter signal. CD3$^-$ cells were gated to plot for CD19$^+$ cells were the phenotype of interest CD24^{hi}CD38^{hi} (Bregs) and the expression of its killer function expressed as CD125$^+$CD178$^+$ and CD125$^-$CD178$^+$ phenotype.*

5. Statistical analysis

For the analysis of the demographic and biochemical parameters an unpaired Student's t-test with a non-parametric Krusal-Wallis test with a Dunns *post hoc* was done using GraphPad prism software version 6 (San Diego, California, USA), The luminex and whole blood flow cytometry data were analyzed using Statistica version 13.4 (StatSoft, Tulsa, Oklahoma, USA). An analysis of variance (ANOVA) with a Fisher LSD *post hoc* test was used to measure difference among the four patient groups. The luminex and whole blood flow cytometry results were represented as LS means with 95% confidence intervals (CI). A P-value <0.05 indicated statistical significance difference and a P ≥0.05 – 0.09. indicates a trend. Qlucore Omics Explorer 3.5 software (Lund, Sweden) was used for the construction and clustering of a heatmap.

Chapter 3

3. Cytokine expression study

Patients with T2D have chronic low-grade inflammation and an increase in pro-inflammatory cytokines. TB patients with T2D have dysregulated immune responses (Podell et al., 2014), resulting in these patients presenting with a greater extend of disease. The aim of this study was therefore to further investigate the immunological imbalance in serum protein levels in individuals with and without TB and T2D, by including a larger number of serum analytes, to better characterize the differences observed in these individuals. Serum samples were collected from HC (n=9), patients with T2D (n=11), TB (n=25) and TB-T2D (n=3) before TB treatment and from patients with TB (n=9) and TB-T2D (n=2) at month two of TB treatment. The serum concentrations of 32 markers were determined using the luminex multiplex immunoassay technology.

3.1 Demographics and clinical characteristics of study participants

Majority of the study participants are females (Table 3.1) except for the TB group that had mostly males. T2D patients have the highest body mass index (BMI) and individuals with a BMI >30Kg/m^2 are defined as obese (WHO), characterizing the T2D patients in this study as obese (Table 3.1). Since BMI will influence cytokine responses, we therefore statistical corrected for BMI and found that the results did not change after correcting for BMI. HbA1c and lipid concentrations were measured in HCs and patients with T2D, in TB-T2D patients at baseline (BL), HbA1c levels were measured before the patients underwent anti-TB treatment. As expected, the HbA1c levels are higher in T2D and TB-T2D patients compared to HCs (Table 3.1).

Table 3. 1: The demographics and clinical characteristics of HC, T2D, TB and TB-T2D patients.

	HC	T2D	TB	TB-T2D	P value
Study Demographics					
Sex (F/M):					
Baseline	6/3	9/2	3/22	2/1	
Month 2	-	-	8/1	2/0	
Age (years):					
Baseline	45.56±11.83^{ab}	54.06±7.46^{a}	34.36±10.84^{b}	43.67±15.28^{ab}	0.0004
Month 2	-	-	31.22±3.73	42.00±21.21	0.82
Clinical Characteristics					
BMI (Kg/m^2)					
Baseline	22.36±4.77^{ab}	31.69±7.88^{a}	18.60±1.87^{b}	21.63±2.48^{ab}	<0.0001
Month 2	-	-	19.63±1.71^{a}	22.45±0.35^{b}	0.04
HbA1c (%)	5.31±0.38^{a}	10.05±2.45^{b}	-	13.77±5.32^{b}	<0.0001
LDL (mmol/L)	2.44±0.74	2.69±1.00	-	-	0.67
HDL (mmol/L)	1.48±0.43	1.42±0.43	-	-	0.87
Triglycerides (mg/dL)	1.62±0.83	1.86±0.98	-	-	0.56
Total cholesterol (mg/dL)	4.66±0.90	4.95±1.19	-	-	0.66

*The values are expressed as mean ± standard deviation. The study participants consisted of HC (n=9), T2D (n=11), TB (n=25) and TB-T2D (n=2) and patients with TB followed up two months into their anti-TB treatment; TB (n=9) and TB-T2D (n=2). The statistical analysis was done using a Mann-Whitney U test to compare two groups and a one-way analysis of variance (ANOVA) with a Bonforoni *post hoc* for comparing more than two groups. Letters a and b indicate statistical significance.

3.2 Cytokine profile differs between patient groups.

The concentration of 32 serum analytes were measured in HCs T2D, TB and TB-T2D patients using the Luminex platform. Unbiased hierarchical clustering of the mean concentration of each analyte in each patient group was done and a heatmap generated using the Qlucore omics explorer software. Eight cytokine clusters were observed (Figure 3.1). Concentrations of MDC, G-CSF, MIP-1b, MMP-3, IL-2, Fas, IL-13, CXCL9/MIG and IL-1β (cluster 1) are higher in HCs compared to the other groups. The concentrations of C4 and complement C4b decrease in the presence of TB (cluster 2). Concentrations of complement C5, C2, C5a and sIL-4R (cluster 3) and sIL-6R, IL-5 and sVEGR3 of cluster 5 are lower in TB-T2D patients in comparison to other groups.

Concentrations of MIP-1α, IL-10 and GCP-2 are higher in T2D patients compared to other groups (cluster 4). T2D patients have low IFN-γ concentrations (cluster 6) while IL-12p40 and eotaxin concentrations are higher in HCs and TB-T2D patients. *Mtb* promotes an increased expression of the serum analytes in cluster 7 (Apo AI, Apo CIII, α1-antitrypsin, IL-2Ra and IP-10) and cluster 8 (SAA, VEGF and CRP). However, the serum markers associated with cluster 7 are mainly higher in TB patients. No statistical significance was found on the concentration of serum analytes in cluster 2 and hence their data will not be shown in the sections to follow.

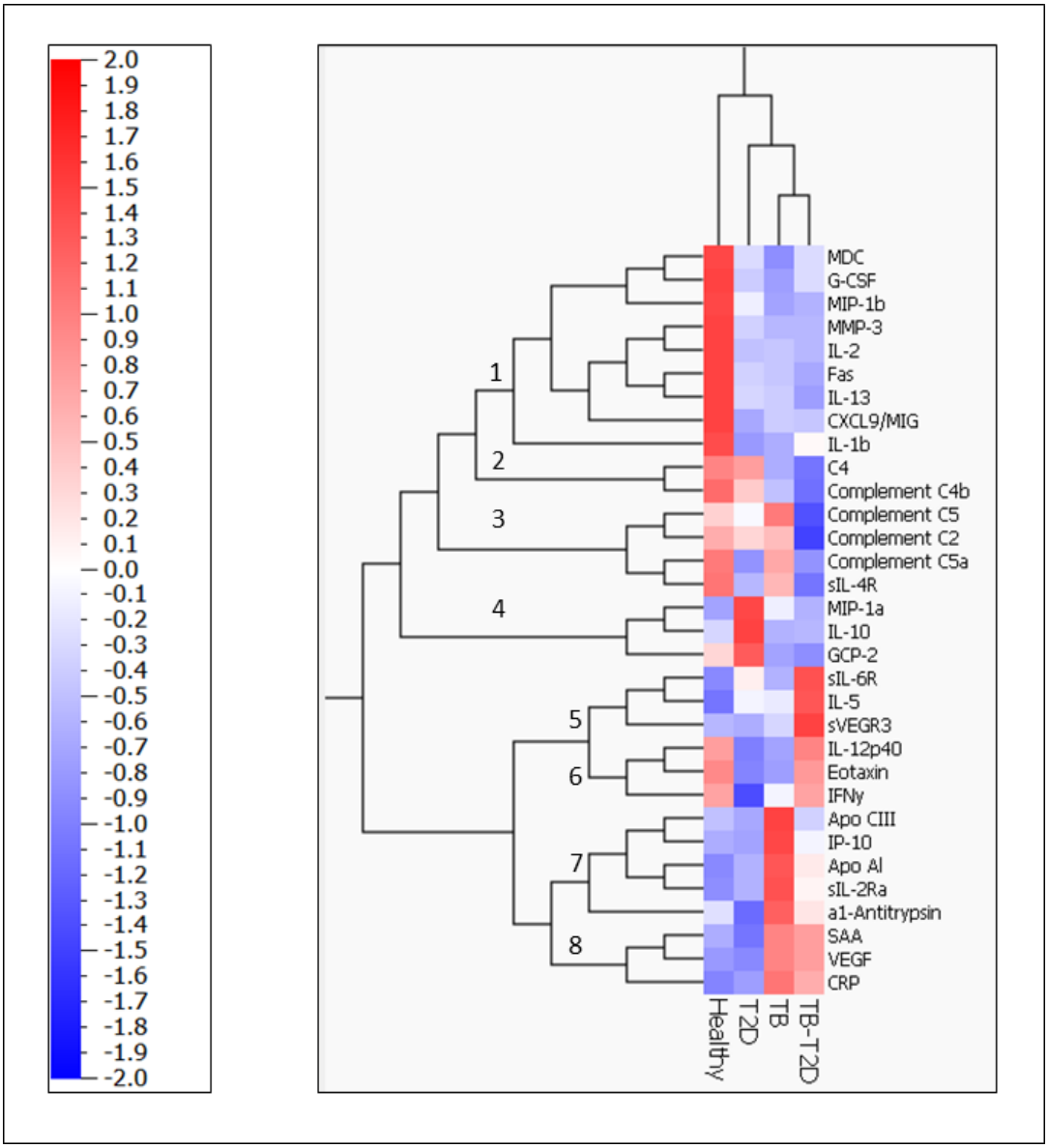

Figure 3.1: Differential expression of 32 serum cytokine concentrations at baseline in the four patient groups. The serum concentrations of 32 analytes were determined in HCs (n=9), T2D (n=11), TB (n=25) and TB-T2D (n=3) patients at BL. There are 8 distinguishable clusters based on the mean concentrations of the serum markers. The colour blue denotes low concentrations of serum markers while the colour red denotes high concentrations of serum markers as shown on the key on the left of the heatmap. The heatmap was generated using the Qlucore Omics explorer software Version 3.5 software.

3.3 Serum concentrations of CXCL9/MIG, IL-2, MDC, MIP-1b and MMP-3 are higher in HCs

In the periphery, the concentration of proteins listed in cluster 1 is higher in HCs (Figure 3.2). Cluster 1 includes cytokines, chemokines and a protease which are all associated with inflammatory responses, cell recruitment, cell damage and cell survival pathways. CXCL9/MIG, IL-2 and MMP-3 are significantly higher in HCs compared to T2D and TB patients. MDC and MIP-1β was significantly higher in HCs compared to TB patients and there was a trend for it to be higher in HCs compared to patients with T2D (p=0.05 and p=0.06, respectively). No significant differences were seen in the other analytes included in this cluster (data not shown).

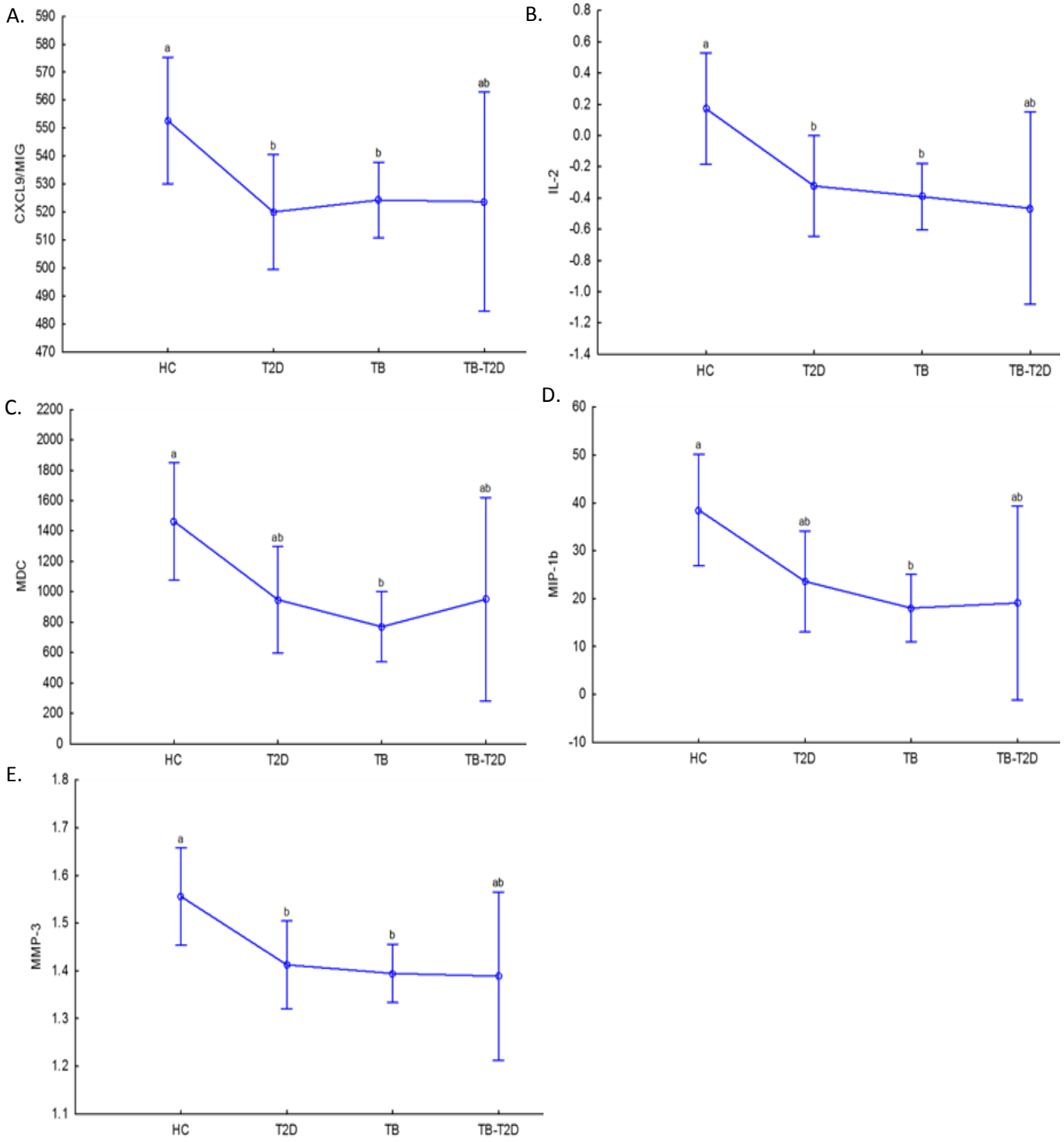

Figure 3.2: Circulating serum concentrations of cytokines included in cluster 1. The mean serum concentrations of CXCL9/MIG (A), IL-2 (B), MDC (C), MIP-1b (D) and MMP-3 (E) were measured in HC (n=9) and patients with T2D (n=11), TB (n=25) and TB-T2D (n=3). Statistical analysis was done using a one-way analysis of variance (ANOVA) with a Fisher LSD post hoc test to compare the differences among groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant differences. Groups sharing letter are not significant difference from each other. The concentrations of the serum analytes are represented in pg/ml.

3.4 The serum concentration of sIL-4R and complement C5a is lower in patients with T2D and TB-T2D.

The complement system consists of proteins with proteolytic properties and upon activation, they induce cell lysis, opsonisation of pathogens and the activation of inflammatory pathways, in efforts to combat and enhance clearance of an invading pathogen. The protein concentration of the C5a in the serum is significantly lower in patients with TB-T2D compared to HCs and TB patients (Figure 3.3). There was a trend for protein concentrations of sIL-4R to be lower in T2D patients (p=0.06) and TB-T2D patients (p=0.09) compared to HCs. No significant differences were found on analytes C5 and C2 included in this cluster (data not shown).

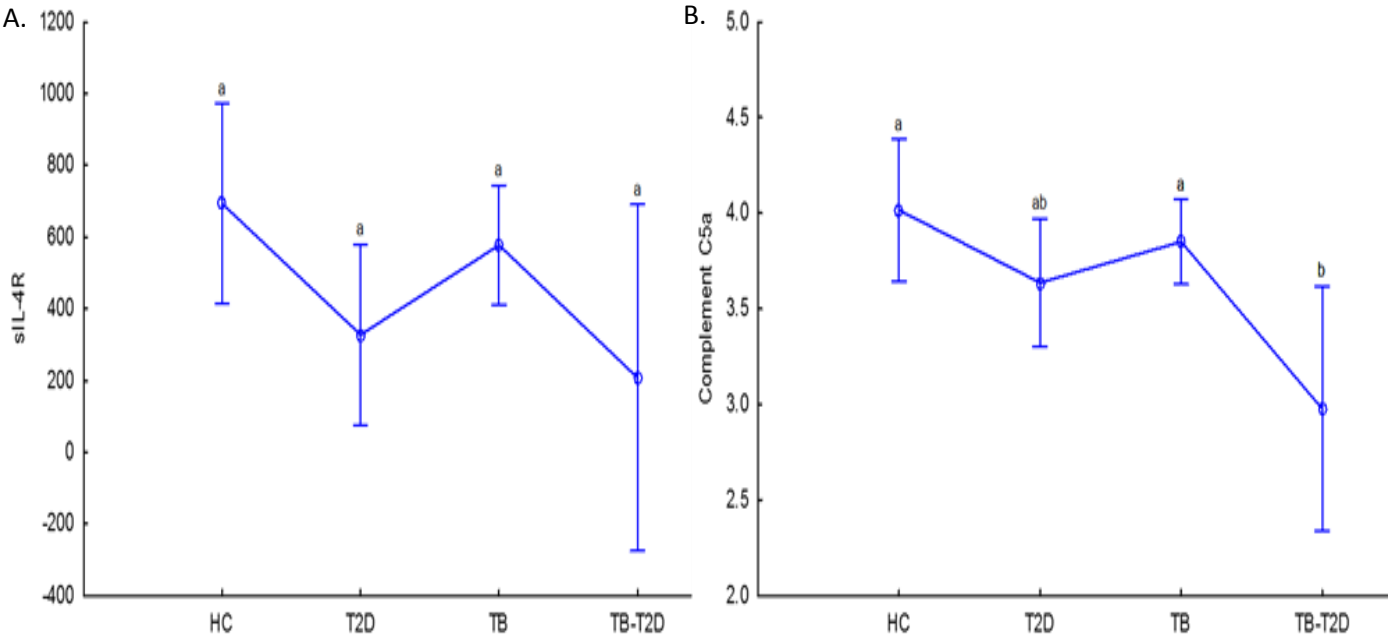

Figure 3.3: Serum concentrations of sIL-4R and complement C5a from which forms part of cytokine cluster 3. The mean serum concentrations of sIL-4R (A) and complement C5a (B) were measured in HCs (n=9) and patients with T2D (n=11), TB (n=25) and TB-T2D (n=3). Statistical analysis was done using a one-way analysis of variance (ANOVA) with a Fisher LSD post hoc test to compare the differences between groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant differences. Groups sharing letter are not significant difference from each other. The concentration of sIL-4R and Complement C5a are represented in pg/ml.

3.5 The serum concentration of IL-10 is higher in patients with T2D.

IL-10 is produced by immune cells such as; T cells, NK cells and APCs. IL-10 can inhibit MHC class II and co-stimulatory molecule expression restricting the migration of pro-inflammatory cytokines and chemokines to lymph node leading to the failure of the recruitment of immune cells to the site of infection (Couper et al., 2008). In T2D patients, the concentration of IL-10 is highest in the T2D group with significance only reached when compared to the TB (Figure 3.4). No significant differences were found on analytes MIP-1α and GCP-2 included in this cluster (data not shown).

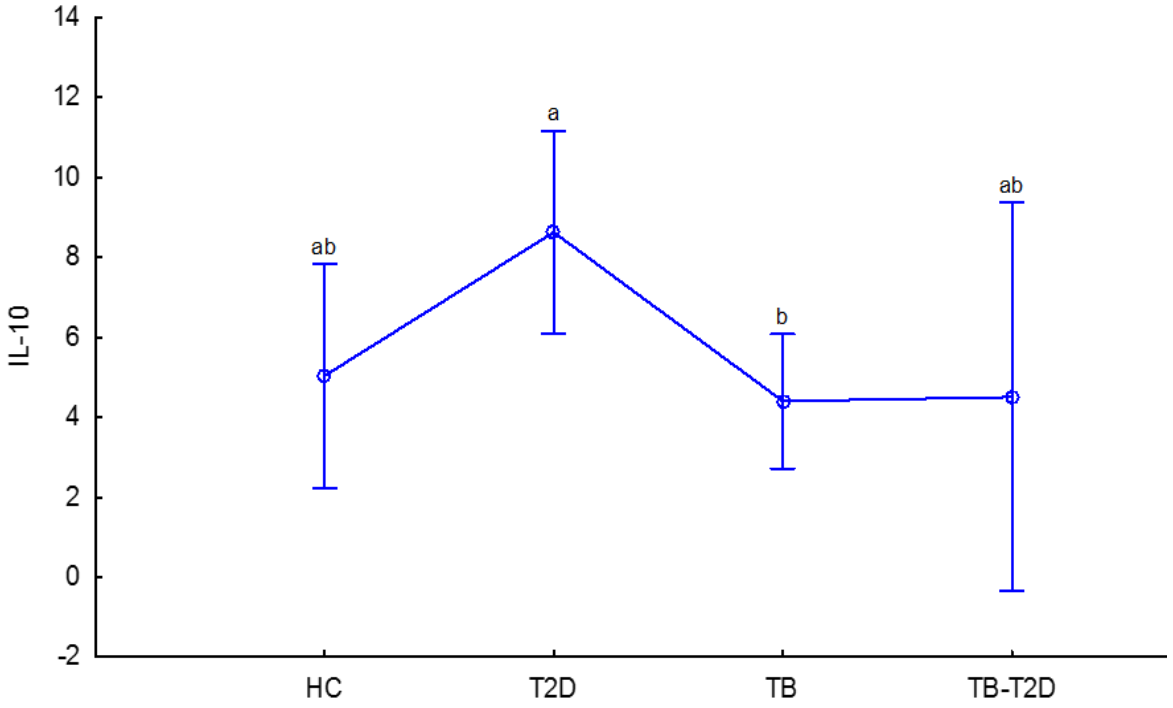

Figure 3.4: The serum concentrations of IL-10 in HCs and patients with T2D, TB and TB-T2D (cluster 4). The mean serum concentration of IL-10 was measured in HCs (n=9) and patients with T2D (n=11), TB (n=25) and TB-T2D (n=3). Statistical analysis was done using a one-way analysis of variance (ANOVA) with a Fisher LSD post hoc test to compare the differences amongst groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference. Groups sharing letter are not significant difference from each other. The concentration of IL-10 is represented as pg/ml.

3.6 The concentrations of sIL-6R and sVEGR3 are higher in patients with TB-T2D.

The serum cytokine concentrations in cluster 5 (Figure 3.1) are higher in TB-T2D patients. The binding of IL-6 to the membrane bound IL-6R modulates inflammatory responses through activating the JAK-STAT pathway (Hurst et al., 2001). In the absence of IL-6R, sIL-6R/IL-6 can activate trans-signalling in cells (Chakraborty et al., 2011).The serum concentration of sIL-6R is significantly higher in TB-T2D patients compared to HCs and TB patients (Figure 3.5A). VEFG are contributory proteins in the lymphatic vascular system during angiogenesis and binds to the VEFG receptor (VEGFR) (Debrah et al., 2006). The soluble VEGFR3 concentration is significantly higher in TB-T2D patients in comparison to all the other groups (Figure 3.5 B). No significant differences were found on the cytokine concentration of IL-5 included in this cluster (data not shown).

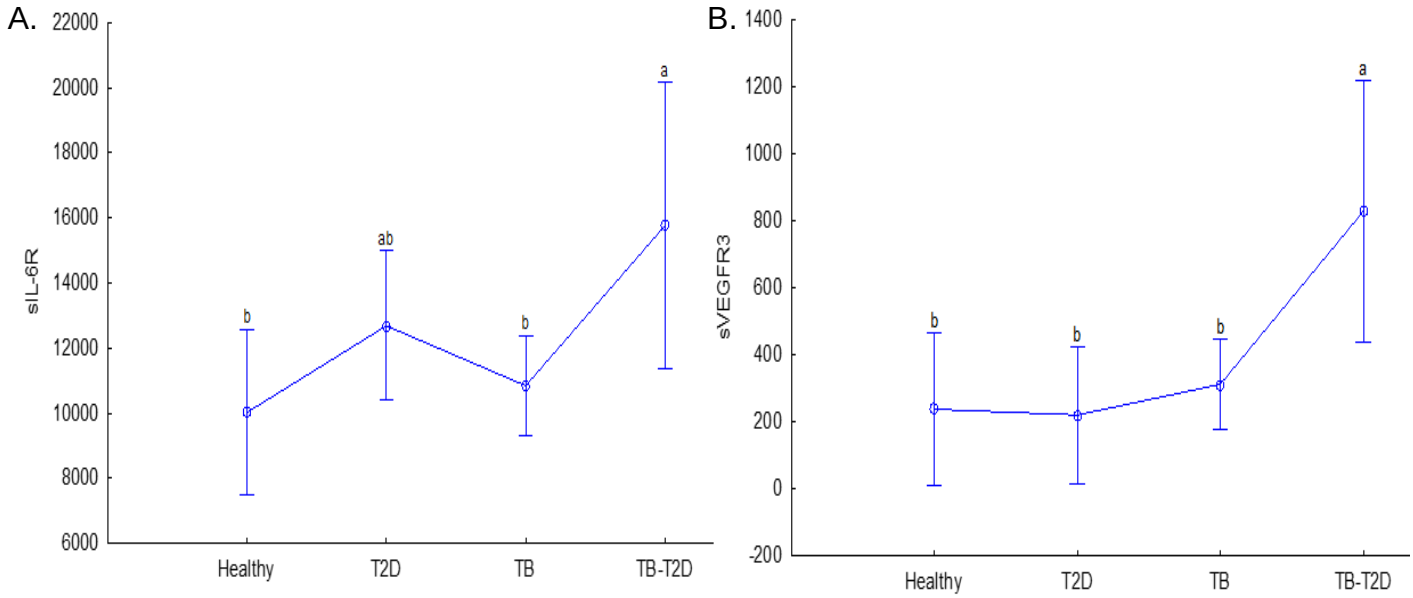

Figure 3.5: Serum concentrations of sIL-6R and sVEFGR3 in HCs and patients with T2D, TB and TB-T2D (cluster 5). The mean serum concentrations were measured in HCs (n=9) and patients with T2D (n=11), TB (n=25) and TB-T2D (n=3). Statistical analysis was done using a one-way analysis of variance (ANOVA) with a Fisher LSD post hoc test to compare the differences between groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference among groups. Groups sharing letter are not significant difference from each other. The concentrations of sIL-6R and sVEGFR3 are represented in pg/ml.

3.7 The serum concentrations of Eotaxin is lower in T2D and TB patients.

Eotaxin, a chemokine facilitating the movement of eosinophils, is significantly lower in patients with T2D and TB compared to HCs and there is a trend for it to be lower in TB-T2D patients (p=0.08) (Figure 3.6). No significant differences were found on cytokines IL-12p40 and IFN-γ included in this cluster (data not shown).

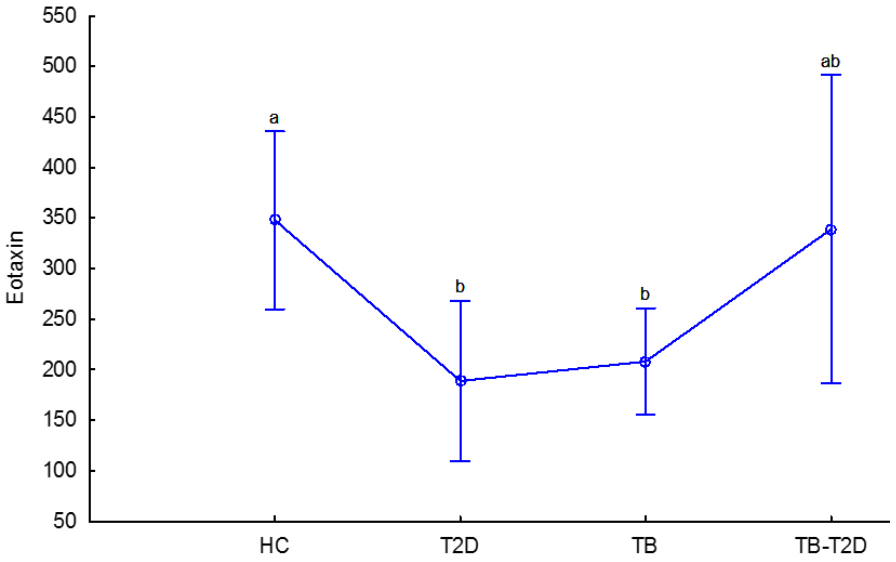

Figure 3.6: The serum concentrations of Eotaxin in HCs and patients with T2D, TB and TB-T2D (cluster 6). The mean serum concentrations of eotaxin were measured in HCs (n=9) and patients with T2D (n=11), TB (n=25) and TB-T2D (n=3). Statistical analysis was done using a one-way analysis of variance (ANOVA) with a Fisher LSD post hoc test to compare the differences among groups. Significant letters are used to indicate significant difference among groups. Results are represented as LS means with 95% confidence intervals. Groups sharing letter are not significant difference from each other. The concentration of Eotaxin is represented as pg/ml.

3.8 Serum concentrations of cytokines in cluster 7 are higher in TB patients.

The serum cytokines in cluster 7 (Figure 3.1) consists of apolipoproproteins (apo) which form covalent bonds with lipids to form lipoproteins. By doing so they are responsible for the regulation and transportation of lipids to different cells in different tissues (Mahley et al., 1984). The serum concentration of Apo AI is significantly higher in TB patients compared to HCs and patients with T2D (Figure 3.7A). It is also higher in TB patients compared to TB-T2D patients, however, it did not reach statistical significance. Apo CIII was significantly higher in TB patients compared to all other groups (Figure 3.7B).

Alpha1-antitrypsin (α1-antitrypsin) is a protease inhibitor responsible for the degradation of elastin an extracellular matrix responsible for the elasticity in tissues.

The degradation of α1-antitrypsin induces inflammation (Hunt and Tuder, 2012). sIL-2Rα is cleaved from the cell membrane through enzymatic processes and competes with the IL-2R for IL-2 resulting in the down regulation of IL-2 mediated immune response (Vanmaris and Rijkers, 2017). Concentrations of both, a1-antitrypsin and sIL-2Rα, are significantly higher in TB patients compared to HCs patients with T2D. No significant differences in the serum concentration of cytokine IP-10 included in this cluster (data not shown).

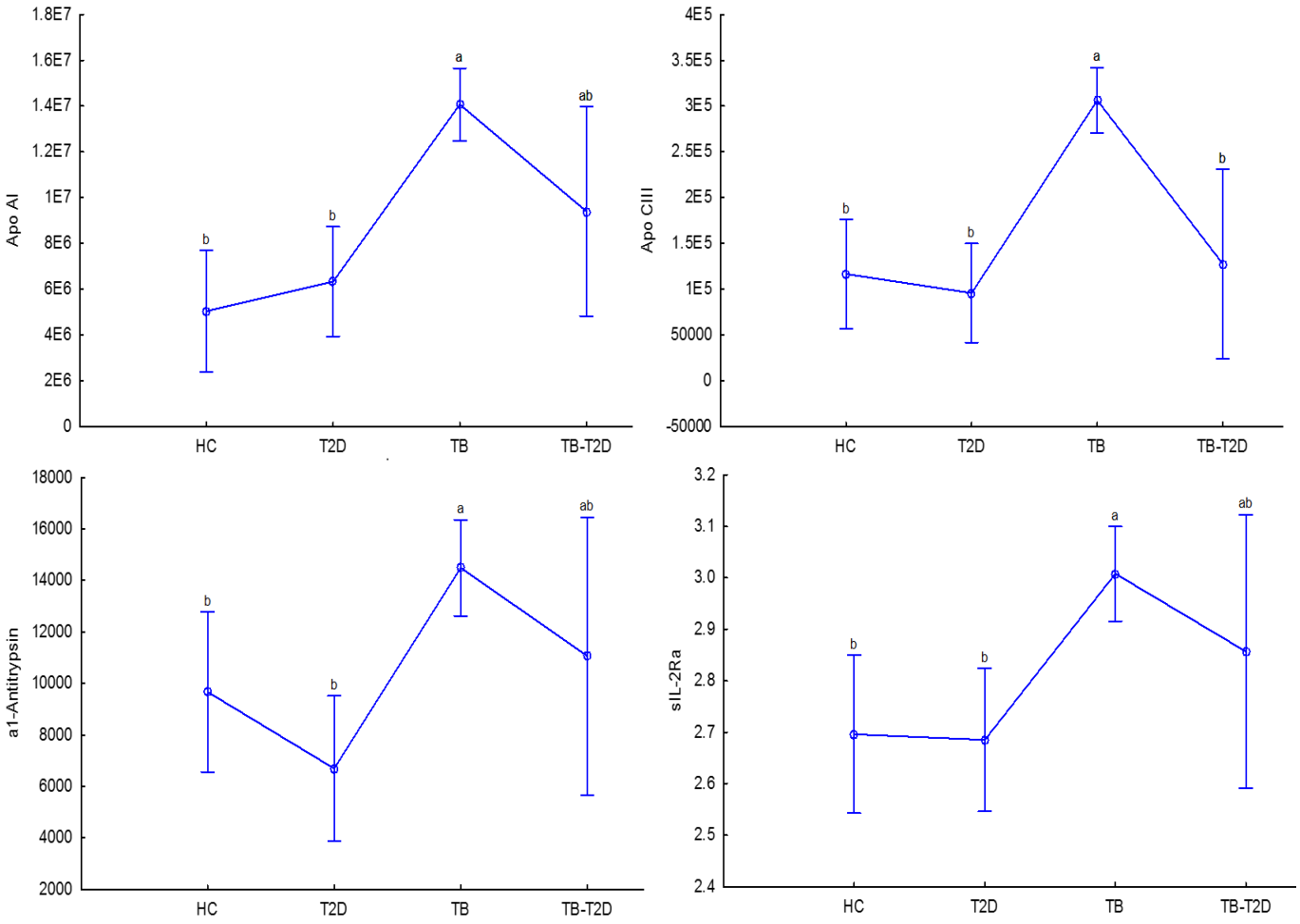

Figure 3.7: The serum concentrations of Apo AI, Apo CIII, α1-antitrypsin and sIL-2Rα in HCs and patients with T2D, TB and TB-T2D (cluster 7). The mean serum cytokine concentrations were measured in HCs (n=9) and patients with T2D (n=11), TB (n=25) and TB-T2D (n=3). Statistical analysis was done using a one-way analysis of variance (ANOVA) with a Fisher LSD post hoc test to compare the differences between groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference among groups. Groups sharing letter are not significant difference from each other. The concentrations of Apo AI, Apo CIII, α1-Antitrypsin are represented in ng/ml and sIL-2Ra concentration is represented as pg/ml.

3.9 The serum concentrations of CRP, SAA and VEGF are higher in TB and TB-T2D patients.

The inflammatory marker C-reactive protein (CRP) is significantly higher in TB and TB-T2D patients than in HCs and T2D patients (Figure 3.8A). SAA is significantly higher in TB patients in comparison to patients with T2D (Figure 3.8B). VEGF concentrations are significantly higher in TB patients compared to HCs and patients with T2D. There is also a TB-T2D patients have higher concentrations than patients with only T2D (Figure 3.8C).

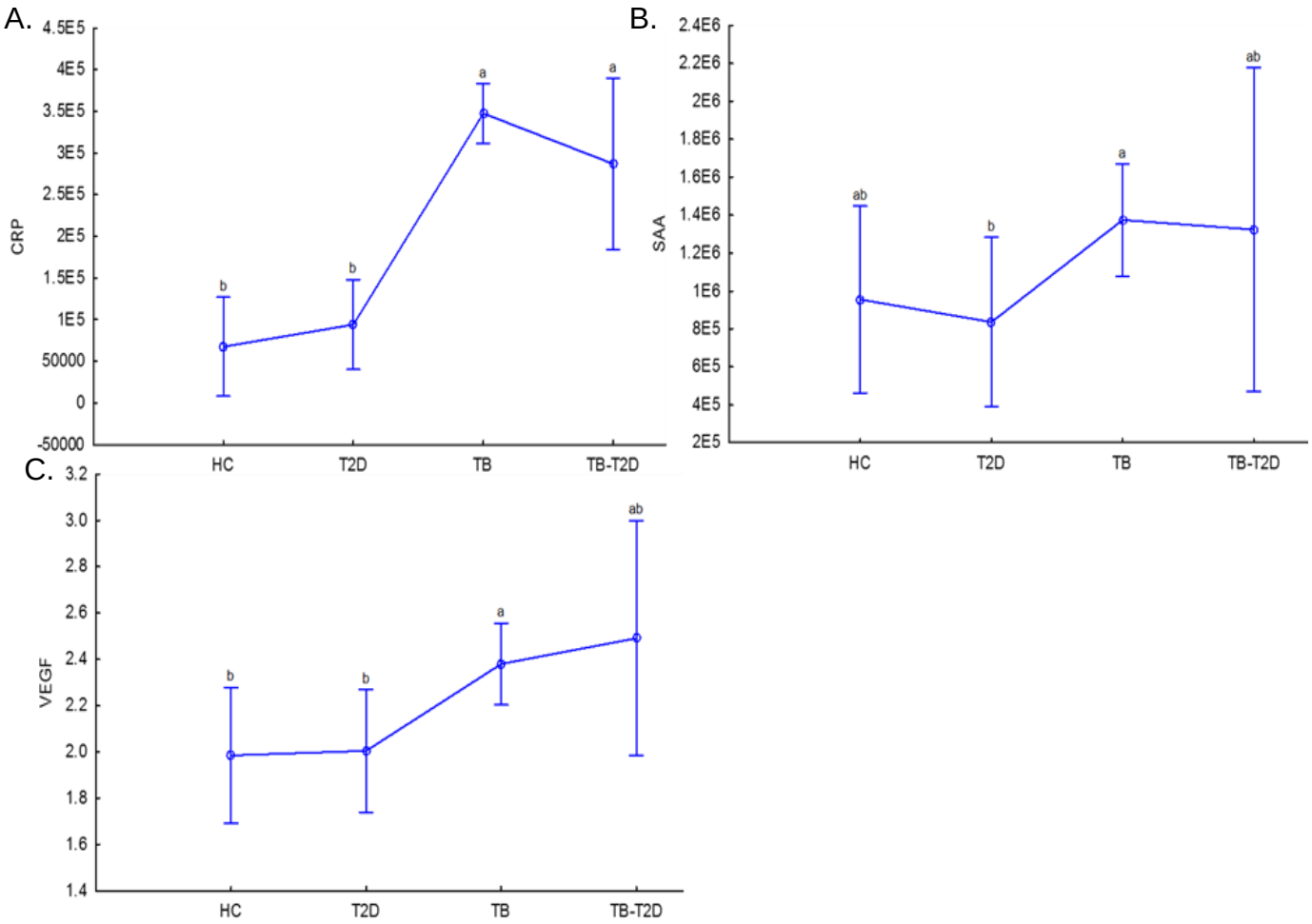

Figure 3.8: The serum concentrations of CRP, SAA, and VEGF in HC, T2D, TB and TB-T2D participants of cluster 8 derived from a heatmap in Figure 3.1. The mean serum cytokine concentrations were measured in HCs (n=9) and patients with T2D (n=11), TB (n=25) and TB-T2D (n=3). Statistical analysis was done using a one-way analysis of variance (ANOVA) with a Fisher LSD post hoc test to compare the differences between groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference among groups. Groups sharing letter are not significant difference from each other. The concentrations of CRP and SAA are represented in ng/ml and VEGF concentration is represented as pg/ml.

3.10 The cytokine profile of differs between TB and TB-T2D patients in response to anti-TB treatment.

The mean concentration of 32 serum analytes were measured in serum samples of TB and TB-T2D patients before initiation of anti-TB treatment and two months into anti-TB treatment. Using the Qlucore Omix explorer software we performed an unbiased hierarchical clustering of the mean concentration of the 32 markers and found five distinguishable clusters (Figure 3.9). The serum concentrations of CRP, IP-10, C4, Complement C4b, α1-antitrypsin, sIL-2Ra, Complement C5a, Apo AI, Apo CIII, IL-13 decrease two months into anti-TB treatment in TB-T2D patients while the concentration levels differ in TB patients at this time-point (cluster 1).

The serum concentrations of SAA and VEGF decrease in TB and TB-T2D in response to anti-TB treatment (cluster 2). There is no treatment response in the serum concentration of analytes in cluster 3 (Figure 3.8). The serum concentrations of sVEGFR3, sIL-6R, G-CSF, IL-1β, IL-5 and IFN-γ decrease in TB-T2D patients in response to anti-TB treatment at month 2. The opposite effect is true for the serum analytes of cluster 5 (Figure 3.8) as their serum concentrations increase in response to treatment. No statistical significance was found on the concentration of serum analytes in cluster 3 and hence their data will not be shown in the sections to follow.

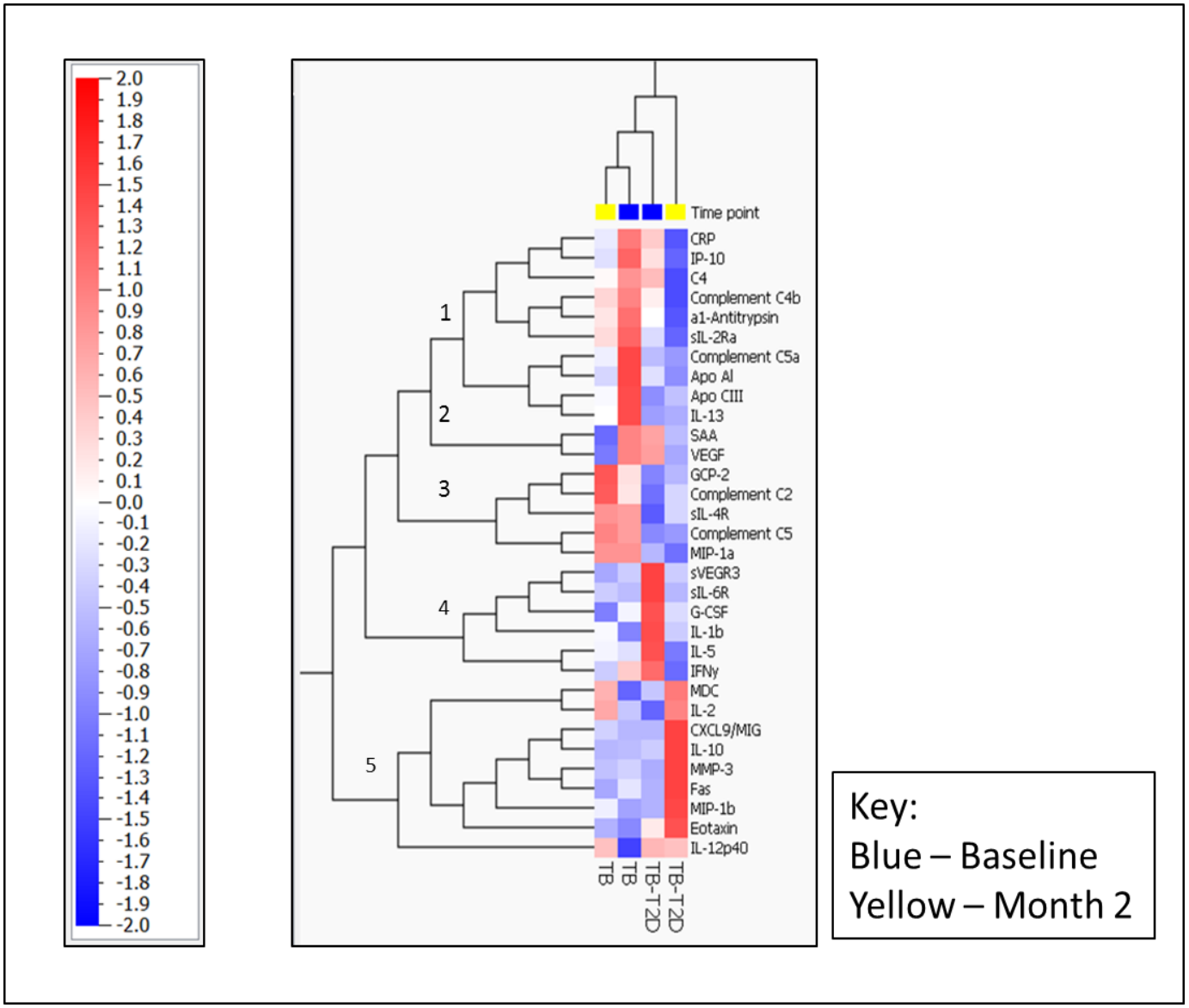

Figure 3.9: Differential expression of 32 serum cytokine concentrations at baseline in the four patient groups. The serum concentrations of 32 analytes were determined in TB BL (n=25), TB-T2D BL (n=3), TB Month 2 (n=9) and TB-T2D Month 2 (n=2) patients at BL. There are 5 distinguishable clusters based on the mean concentrations of the serum markers. The colour blue denotes low concentrations of serum markers while the colour red denotes high concentrations of serum markers as shown on the key on the left of the heatmap. The heatmap was generated using the Qlucore Omics explorer software Version 3.5 software.

3.11 Serum concentrations of Apo AI, Apo CIII, α1-Antitrypsin, CRP and C5a in cluster 1 change during the first two months of anti-TB treatment in TB patients.

The serum concentrations of Apo AI, Apo CIII, α1-antitrypsin and CRP significantly decrease from baseline to month 2 in TB patients, but not TB-T2D (Figure 3.10 A-D). Apo CIII and C5a concentrations are significantly lower in TB-T2D patients compared to TB patients at baseline (Figure 3.10 B and E). No significant differences were found on analytes, IP-10, C4b, sIL-2Ra and IL-13 included in this cluster (data not shown).

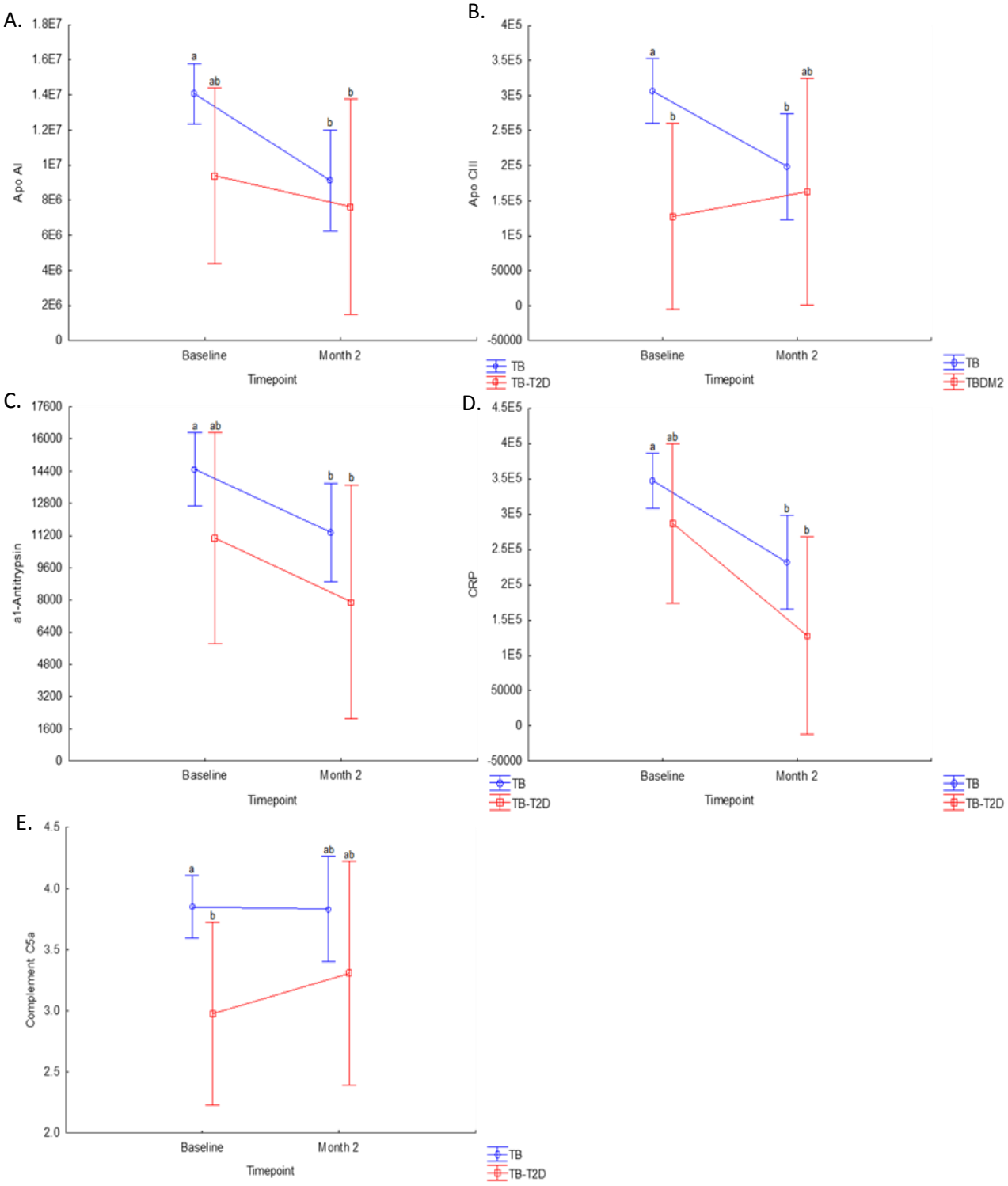

Figure 3.10: The serum concentrations of Apo AI, Apo CIII, α1-antitrypsin, CRP and complement C5a (A-E) at baseline and two months into anti-TB treatment, cluster 1 derived from a heatmap in Figure 3.9). The mean concentration for TB (n=25) and TB-T2D (n=3) at baseline and TB (n=9) and TB-T2D (n=2) patients, month 2 into TB treatment. Statistical analysis was done using a one-way analysis of variance (ANOVA) with a Fisher LSD post hoc test to compare the differences between groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference among groups. Groups sharing letter are not significant difference from each other. The serum concentrations of Apo AI, Apo CIII, α1-antitrypsin and CRP were represented in ng/ml and Complement C5a in pg/ml.

3.12 The serum concentrations of VEGF decrease in TB and TB-T2D patients in response to treatment

On the heatmap in Figure 3.9 VEGF is part of cluster 2 and based on the cluster the concentrations are higher before TB and TB-T2D patients undergo treatment and decrease two months into treatment (Figure 3.11). There is only statistically significance in TB patients. No significant differences were found on SAA included in this cluster (data not shown).

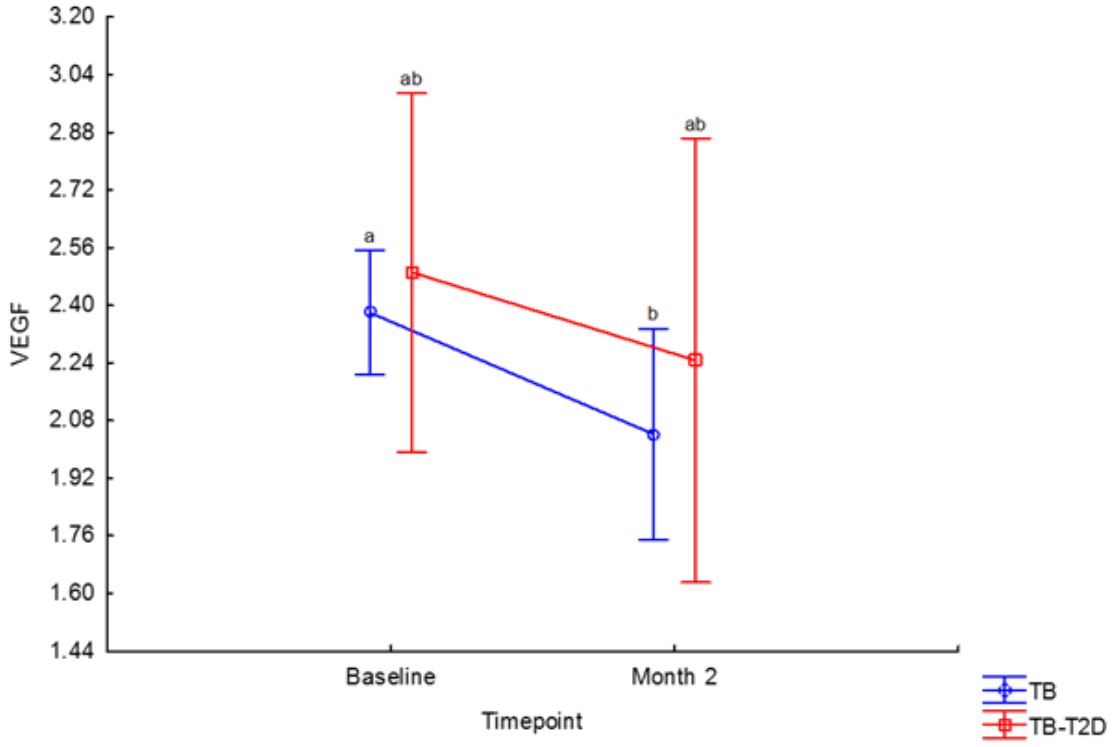

Figure 3.11: The serum concentration VEGF at baseline and two months into anti-TB treatment, cluster 2 derived from a heatmap in Figure 3.9).VEGF concentrations were measured in TB (n=25) and TB-T2D (n=3) at baseline and TB (n=9) and TB-T2D (n=2) patients two months into anti-TB treatment. Statistical analysis was done using a one-way analysis of variance (ANOVA) with a Fisher LSD post hoc test to compare the differences between groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference among groups. Groups sharing letter are not significant difference from each other. The serum concentration of VEGF is represented in pg/ml.

3.13 The serum concentrations of the analytes included in cluster 4 are higher in patients with TB-T2D compared to TB patients at baseline.

At the initiation of TB treatment, sVEGFR3 and sIL-6R concentrations are higher in patients with TB-T2D compared to patients with TB (Figure 3.12 A and B). During the first two months of TB treatment, sIL-6R levels decrease in the TB-T2D group to levels similar to those observed in TB patients. sIL-6R levels remain unchanged, during this period, in patients with TB. No significant differences were observed in IL-5 and IL-1β concentrations (Figure 3.12 C and D). There are however trends suggesting IL-1β concentrations are higher in TB-T2D patients at baseline ($p=0.09$) and month 2 ($p=0.07$) compared to TB patients. The trends indicate that are higher IL-1β concentrations in TB-T2D patients before anti-TB treatment. No significant differences were found in the concentration of G-CSF and IFN-γ included in this cluster (data not shown).

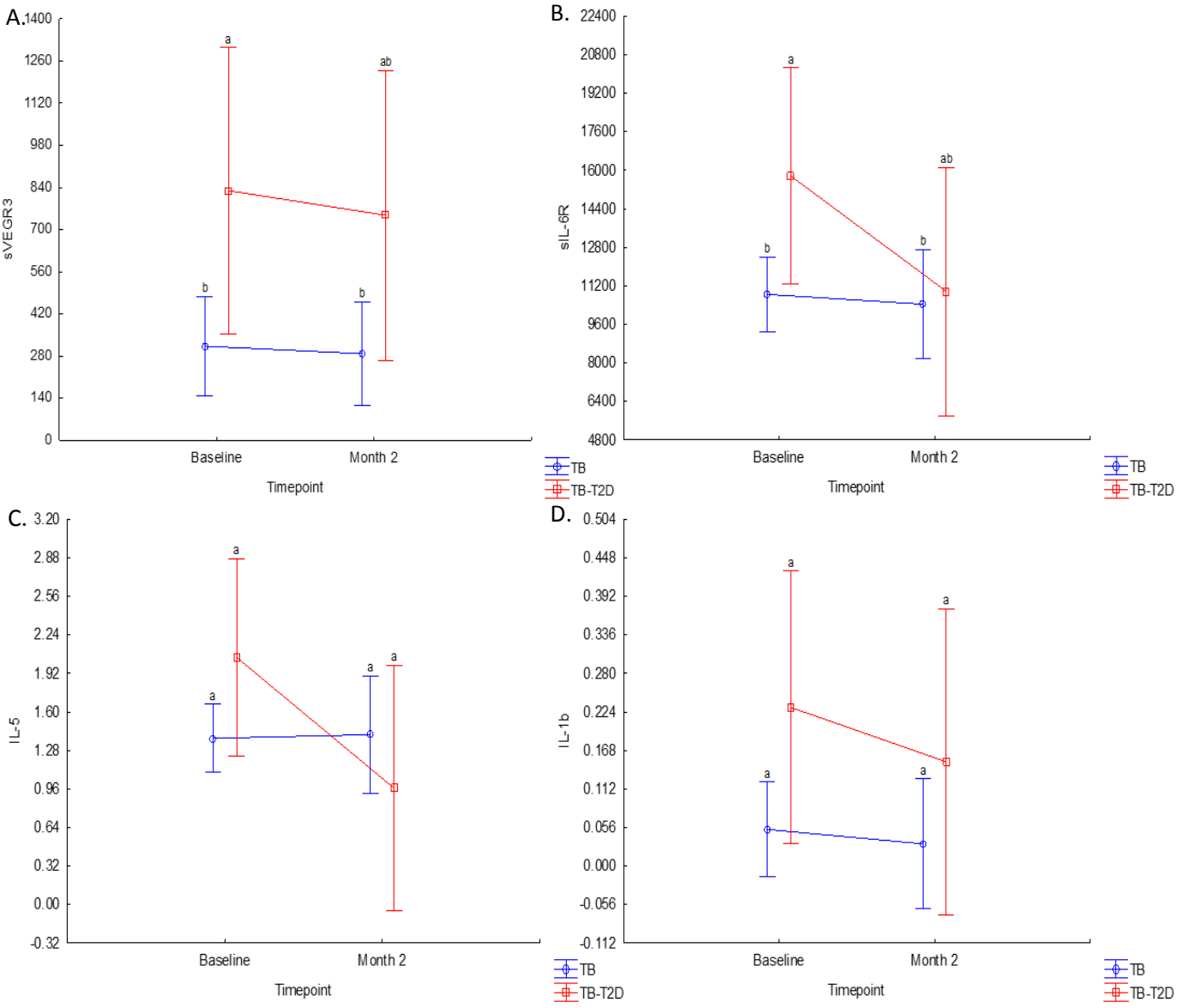

Figure 3.12: The serum concentrations of sVEGFR3, sIL-6R, IL-5 and IL-1β (A-D) at baseline and two months into anti-TB treatment, cluster 4 derived from a heatmap in Figure 3.9).The mean concentration of TB (n=25) and TB-T2D (n=3) at baseline and TB (n=9) and TB-T2D (n=2) patients two months into anti-TB treatment. Statistical analysis was done using a one-way analysis of variance (ANOVA) with a Fisher LSD post hoc test to compare the differences between groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference among groups. Groups sharing letter are not significant difference from each other. The serum of concentrations of sVEGFR3, sIL-6R, IL-5 and IL-1β were represented as pg/ml.

3.14 The serum concentrations of CXCL9/MIG, Eotaxin, MIP-1β and MMP-3 are higher in TB-T2D patients at month two of TB treatment.

The serum concentrations of CXCL9/MIG, MIP-1β and MMP-3 change significantly over time in the TB-T2D group, but not in the TB group (Figure 3.13). There is no significant difference in Eotaxin concentrations in the TB-T2D group (Figure 3.13 B), however, a similar trend in response is observed as the latter mentioned serum analytes. The increase in the cytokine and chemokine concentrations is an indication of a treatment response in the TB-T2D group. There were no significant differences observed in the TB group during treatment. No significant differences were found in the concentration of MDC, IL-2, IL-10, Fas and IL-12p40 included in this cluster (data not shown).

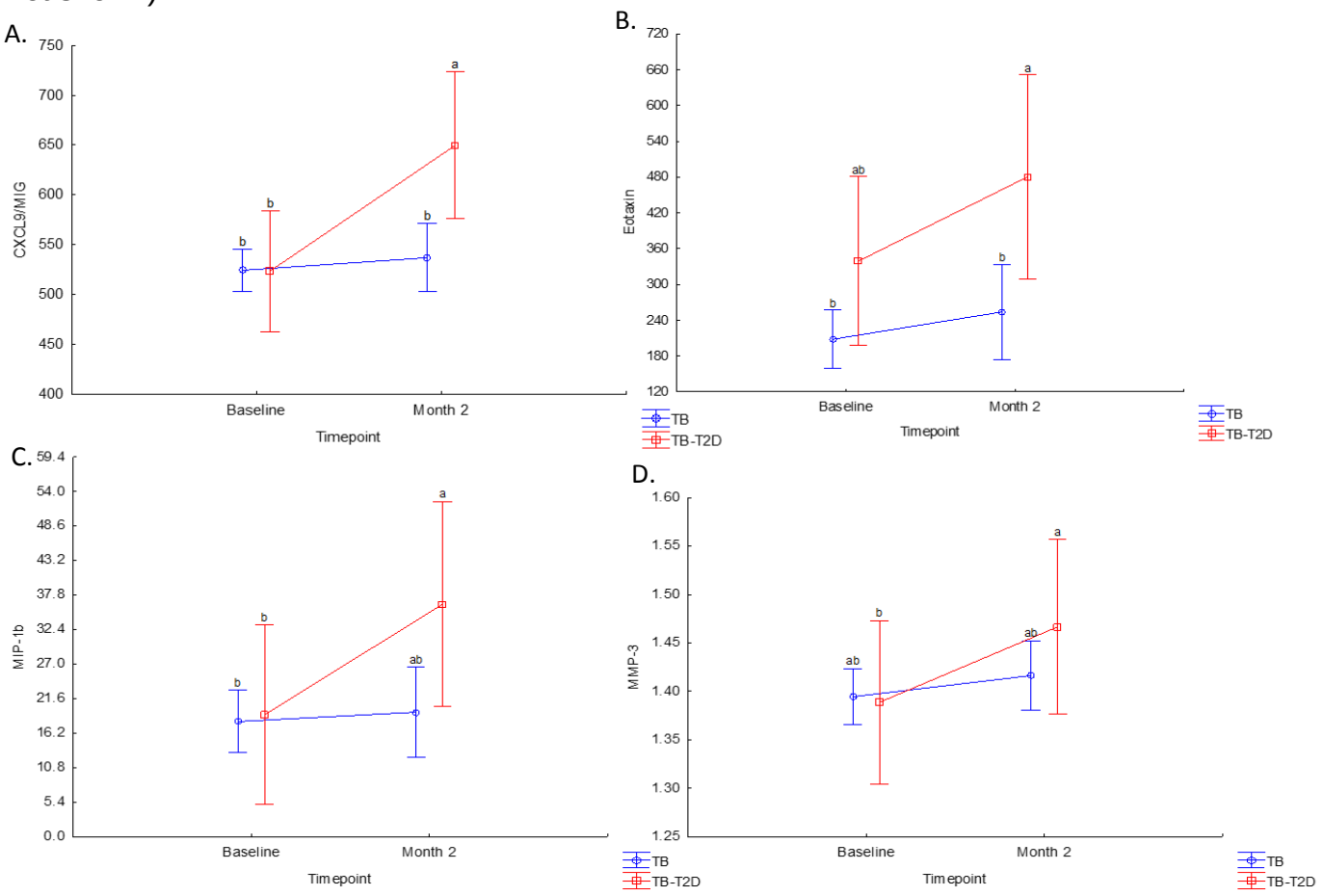

Figure 3.13: The serum concentrations of CXCL9/MIG, Eotaxin, MIP-1β and MMP-3 (A-D) at baseline and two months into anti-TB treatment, cluster 5 derived from a heatmap in Figure 3.9).The mean concentration of TB (n=25) and TB-T2D (n=3) at baseline and TB (n=9) and TB-T2D (n=2) patients two months into anti-TB treatment. Statistical analysis was done using a one-way analysis of variance (ANOVA) with a Fisher LSD post hoc test to compare the differences between groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference among groups. Groups sharing letter are not significant difference from each other. The serum concentrations of CXCL9/MIG, Eotaxin, MIP-1β and MMP-3 were represented in pg/ml.

3.15 Serum proteins correlate with lipid concentrations in HCs with and without T2D.

We have shown that dyslipidaemia in different ethnic groups are driven by different lipids (Restrepo et al., 2018). Although, we did not observe significant differences in lipid concentration of HC and T2D patient. Patients with T2D still had higher LDL, triglycerides and cholesterol therefore we were interested to investigate if the serum markers correlated with the lipid concentrations. The serum concentrations of Apo CIII and α1-antitrypsin are negatively correlated with triglyceride concentrations (Figure 3.14 A and B). sIL-6R concentrations are positively correlated to LDL and cholesterol concentrations (Figure 3.14 C and D). There is also a negative correlation between IL-12p40 and HDL (Figure 3.14 E).

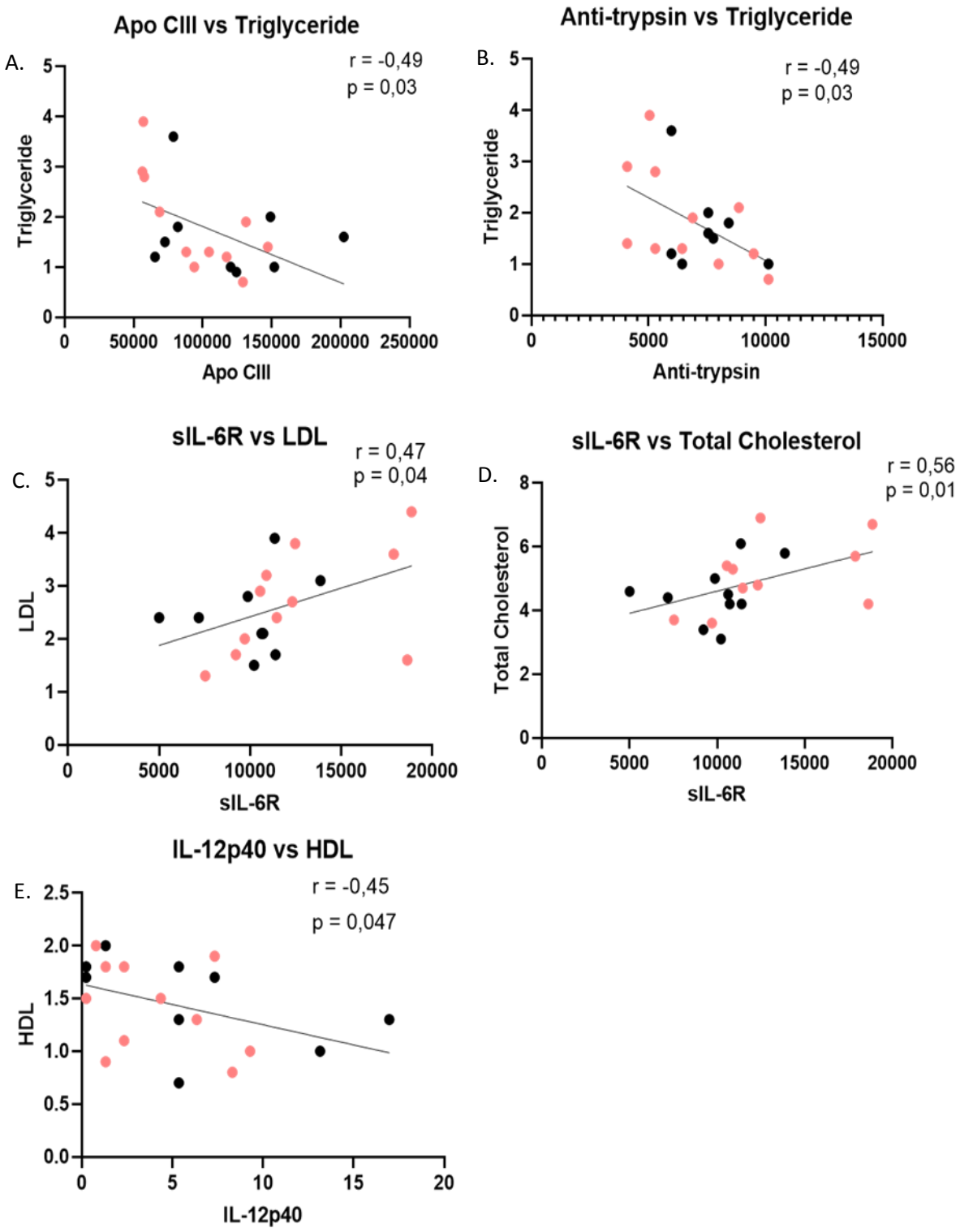

Figure 3.14: Correlations between serum concentration analytes and periphery lipid concentration between the HC and T2D groups. The correlation denotes the relationship between the serum lipid and protein concentrations in the HCs (n=9) and patients with T2D (n=11). Significant correlations were observed in (A) Apo CIII and Triglycerides, (B) α1-antitrypsin and Triglycerides, (C) sIL-6R and LDL (D) sIL-6R and Total cholesterol and (E) sIL-6R and HDL. Black dots represent HC group and red dots represent the T2D group. Correlations were determined using a Spearman Rank correlation.

3.16 Conclusion

We have identified interesting clusters of cytokines and host serum immune markers even though some of them are not significantly different, this will aid in identifying pathways in which the clusters are involved. This will help us understand why T2D patients are more susceptible and why TB patients with T2D present have a greater extent of disease. It is worth noting our results are limited a small sample size especially in the TB-T2D group and we did not investigate the extent of disease in these participants.

There was no statistical differences in lipid concentrations, they however did correlate with serum cytokine concentrations. Our correlation analysis did not statistical differentiate between the two study groups (HC and T2D), so our correlations are reported as an overall correlation. Investigating the immunological pathways in which the different clusters of cytokines are involved in could aid in understanding which pathways are involved in the disease progression in TB and TB-T2D patients. If different pathways are involved one could then speculate that it might play a role in susceptibility and in the extent of disease. We have not looked into their chest x-rays and we did not follow the patients until the end of treatment hence we can only speculate on the extent of disease.

Chapter 4

4. Phenotyping the Whole Blood of TB patients with or without T2D

The frequencies and function of immune cells in TB patients with T2D are altered (Lönnroth et al., 2010; Raposo-García et al., 2017) leaving them vulnerable to increased *Mtb* replication and increased severity of TB disease. Currently, majority of the research investigates the immunological aspects of TB patients with T2D in relation to their role in T-cells and macrophages. Preliminary data obtained from our group should that TB patients with T2D have increased absolute B cells in comparison to TB patients without T2D. The role of B cells as contributors of adaptive immunity in TB-T2D remains largely understudied. Therefore, the aim of this chapter is to determine the frequency of regulatory and killer B cells by investigating the expression of FasL ($CD178^{+}$) and IL-5Rα ($CD125^{+}$) with the use of flow cytometry. For this study, 54 whole blood samples were collected from HCs (n=6), patients with T2D (n=19), TB (n=14) and TB-T2D (n=3) before treatment and patients with TB (n=12) at months two of anti-TB treatment.

4.1 Demographics and clinical characteristics of study participants

Fifty-four participants were enrolled across all four patient groups for the phenotypic analysis of B cell. Patients with T2D are obese and TB patients are underweight at the initiation of treatment (Table 4.1). As highlighted in chapter 3 section 3.1, BMI was corrected for and no statistical differences were observed. Patients with T2D have a higher HbA1c. There was no statistically significant difference in lipid concentrations between HC and T2D groups expect for higher concentrations in the T2D group. The clinical characteristics of the participants are listed in Table 4.1.

Table 4 1: Demographics and clinical characteristics of HCs, T2D patients and TB patients with and without T2D.

	HC	T2D	TB	TB-T2D	P value
Study Demographics					
Sex (F/M):					
Baseline	5/1	14/5	2/12	0/3	
Month 2	-	-	4/8	-	
Age (years):					
Baseline	49.83±9.22^{ab}	52.05±8.70^{a}	38.50±10.84^{b}	41.00±11.27^{b}	0.0002
Month 2	-	-	36.25±8.98	-	
Clinical Characteristics					
BMI (Kg/m^2)					
Baseline	22.52±4.75^{ab}	31.51±6.55^{a}	18.41±2.67^{b}	21.07±2.64^{b}	<0.0001
Month 2	-	-	19.58±2.24	-	
HbA1c (%)	5.27±0.44^{a}	10.02±2.02^{b}	-	12.83±6.10^{b}	0.0008
LDL (mmol/L)	2.50±0.78	2.75±0.78	-	-	0.46
HDL (mmol/L)	1.72±0.23	1.26±0.36	-	-	0.01
Triglycerides (mg/dL)	1.23±0.35	2.05±1.31	-	-	0.10
Total cholesterol (mg/dL)	4.77±0.72	4.94±0.94	-	-	0.52

*The values are expressed as mean ± standard deviation. P-value <0.05 was considered as significant and the statistical analysis was using a Mann-Whitney U test for the comparison of two groups and a one-way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* for the comparison of more than two groups. Letters a-c indicate statistical significance. HbA1c (Glycated haemoglobin), HDL (High density lipoprotein) and LDL (Low density lipoprotein).

4.2 CD4$^+$ and CD8$^+$ T cell frequencies in the different patient groups.

CD3$^+$ T cells were gated from the lymphocytic population and CD4$^+$ and CD8$^+$ T cells were gated from CD3$^+$ T cells. An increased frequency of CD3$^+$ T cells is present in the HCs compared to TB patients and T2D patients compared to TB patients (p=0.08), however these differences did not reach statistical significance (Figure 4.1A). Subsequently the distribution of activated (CD38$^+$) and nonactivated (CD38$^-$) CD4$^+$ and CD8$^+$ T cell subsets were examined. As expected, a greater frequency of CD4$^+$ vs CD8$^+$ T cells were observed in all four study groups. The frequency of CD4$^+$ T cells is however the highest in patients with TB-T2D and the CD8$^+$ T frequency lowest in HCs (Figure 4.1B). The percentage of activated CD4$^+$ and CD8$^+$ T cells are higher HCs and TB patients, respectively.

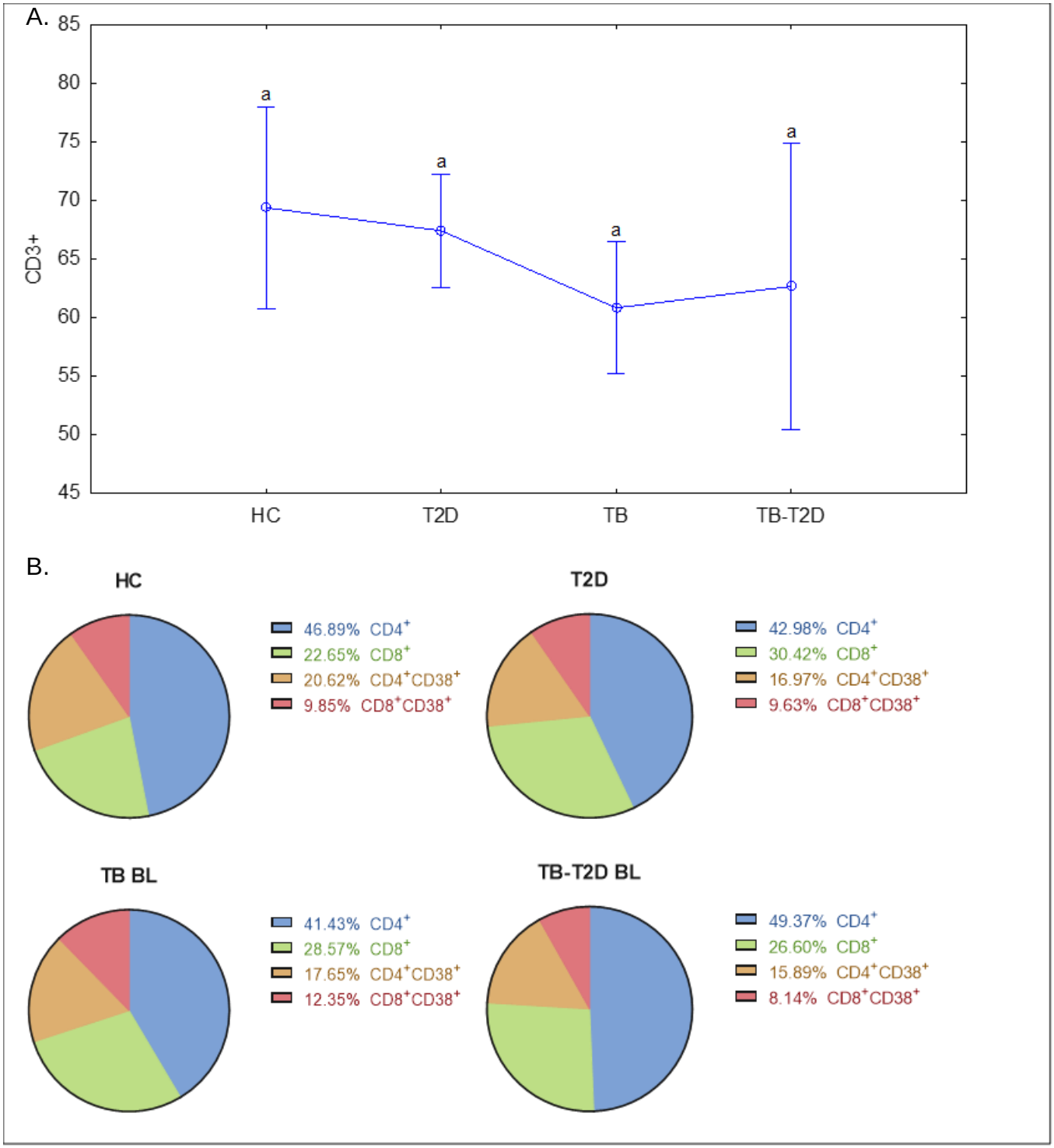

Figure 4.1: The distribution and frequencies of CD4⁺ and CD8⁺ T cells in HCs and patients with T2D, TB and TB-T2D prior to initiation of anti-TB treatment. A) A lymphocyte gate was used to gate for CD3⁺ T cells in WB samples. The mean frequency of CD3⁺ T cells are represented in (%). B) Four pie charts illustrate the distribution of CD3⁺, CD4⁺ and CD8⁺ T cells as well as activated T cells expressing CD38 frequencies (%) in HC (n=6), T2D (n=19), TB (n=14) and TB-T2D (n=3) before anti-TB treatment. Pie charts were constructed on Graphpad Prism version 8. Statistical analysis was done using ANOVA with a Fisher LSD post hoc test to compare the differences amongst groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference among groups. Groups sharing letter are not significant difference from each other.

4.3 The frequency of memory B cells is lower in TB patients before initiation of anti-TB treatment

In chapter 1 we highlighted the contribution of B cells in TB immunity as through cytokine production, presentation of *Mtb* antigens to naïve T cells and their production of antibodies. Joosten et al showed B cell subset frequencies were altered during TB disease and we want to investigate them in presence of T2D. One of the investigated subsets was memory B cells and they have a highly regulated memory immune cells are an important aspect of the immune system, providing a rapid and effective immune response upon a re-encounter with a specific antigen. We investigated the frequency of resting memory B cells and their activation state in the four patient groups. T2D patients have a higher frequency of resting memory B cells ($CD19^{+}CD27^{+}$) while TB patients have the lowest frequency (Figure 4.2B). T2D and TB patients have a lower percentage of activated memory B cells compared to HCs and TB-T2D patients (Figure 4.2A). HCs ($p=0.09$) and patients with T2D ($p=0.09$) have a higher frequency of activated memory B cells than TB patients although not statistically significant (Figure 4.2B).

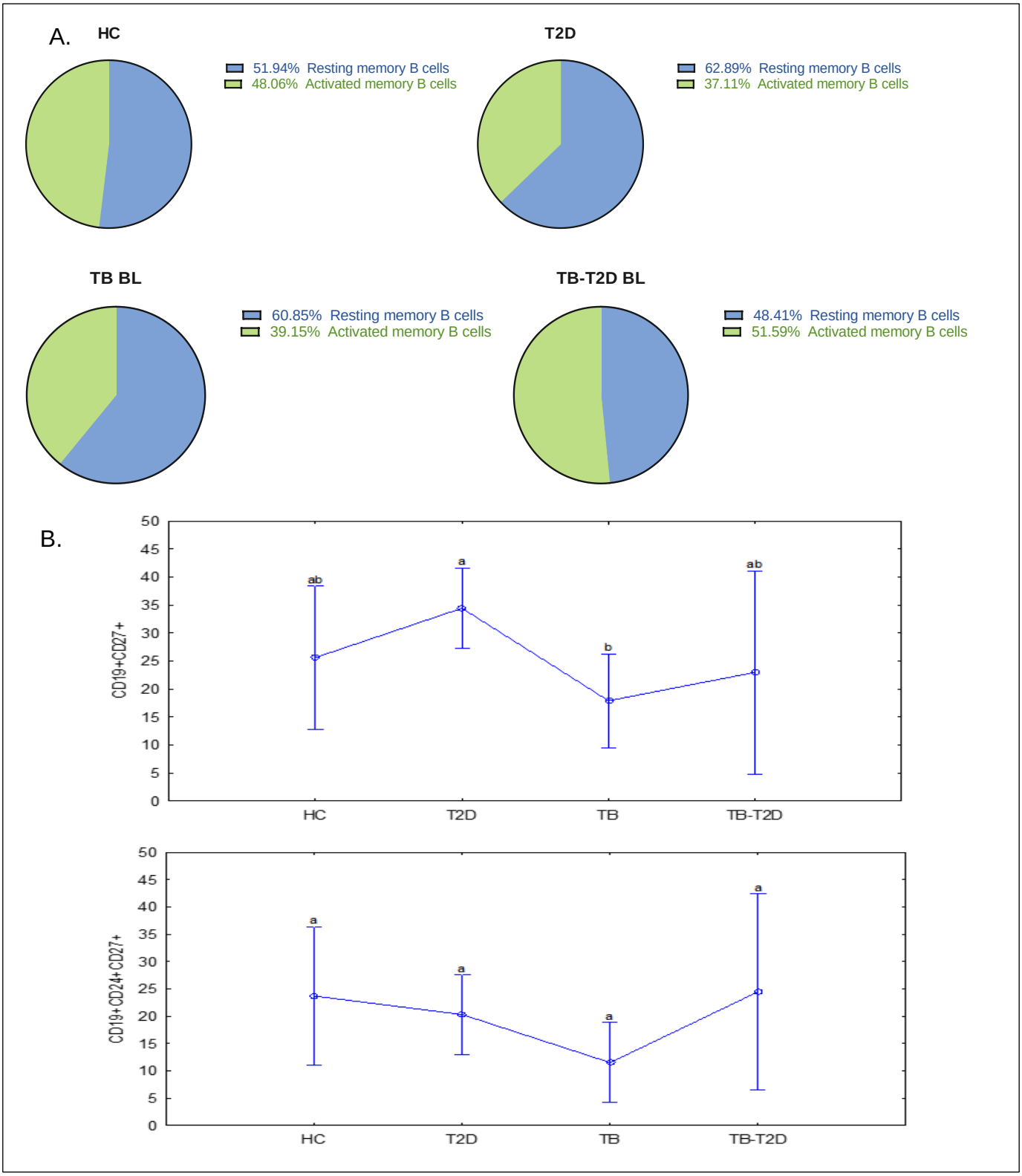

Figure 4.2: The distribution and frequencies of resting and activated memory B cells in the four different patient groups before initiation of anti-TB treatment. A**)** Pie charts B) Line graphs the frequencies (%) of resting memory B cells $CD19^{+}CD27^{+}$ and activated memory B cells $CD19^{+}CD24^{+}CD27^{+}$ in HCs (n=6) and patients with T2D (n=19), TB (n=14) and TB-T2D (n=3) before initiation of anti-TB treatment. A lymphocyte, gated $CD3^{-}$ cells and $CD19^{+}$ from the negative gate. Pie charts were constructed on Graphpad Prism version 8. Statistical analysis was done using ANOVA with a Fisher LSD post hoc test to compare the differences amongst groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference among groups. Groups sharing letter are not significant difference from each other.

4.4 Naïve B cells of TB patients have an increased expression of apoptotic inducers IL-5Rα and FasL

The Fas/FasL (CD178) pathway induces cell death and the surface expression of IL-5Rα (CD125) enhances the induction of apoptosis through the Fas/FasL pathway (Klinker et al., 2013). CD125 and CD178 are expressed by all immune cells. Our group identified that the expression of FasL and IL-5Rα was upregulated in B cells at the end of TB treatment (van Rensburg et al., 2016). Naïve B cell frequencies are higher in TB-T2D patients compared to TB patients (Figure 4.3A). Although not statistically significant, it is in line with what we found in absolute B cell numbers in these patients (manuscript in preparation).There are, however, no significant differences in B cells expressing CD178 in the absence of CD125 in all four study groups (CD19+CD125-CD178+; Figure 4.3B).TB patients have a significantly higher frequency of B cells expressing both the apoptotic inducers CD125 (IL-5Rα) and CD178 (FasL) when compared to HCs and T2D patients (CD19+CD125+C178+ (Figure 4.3C).

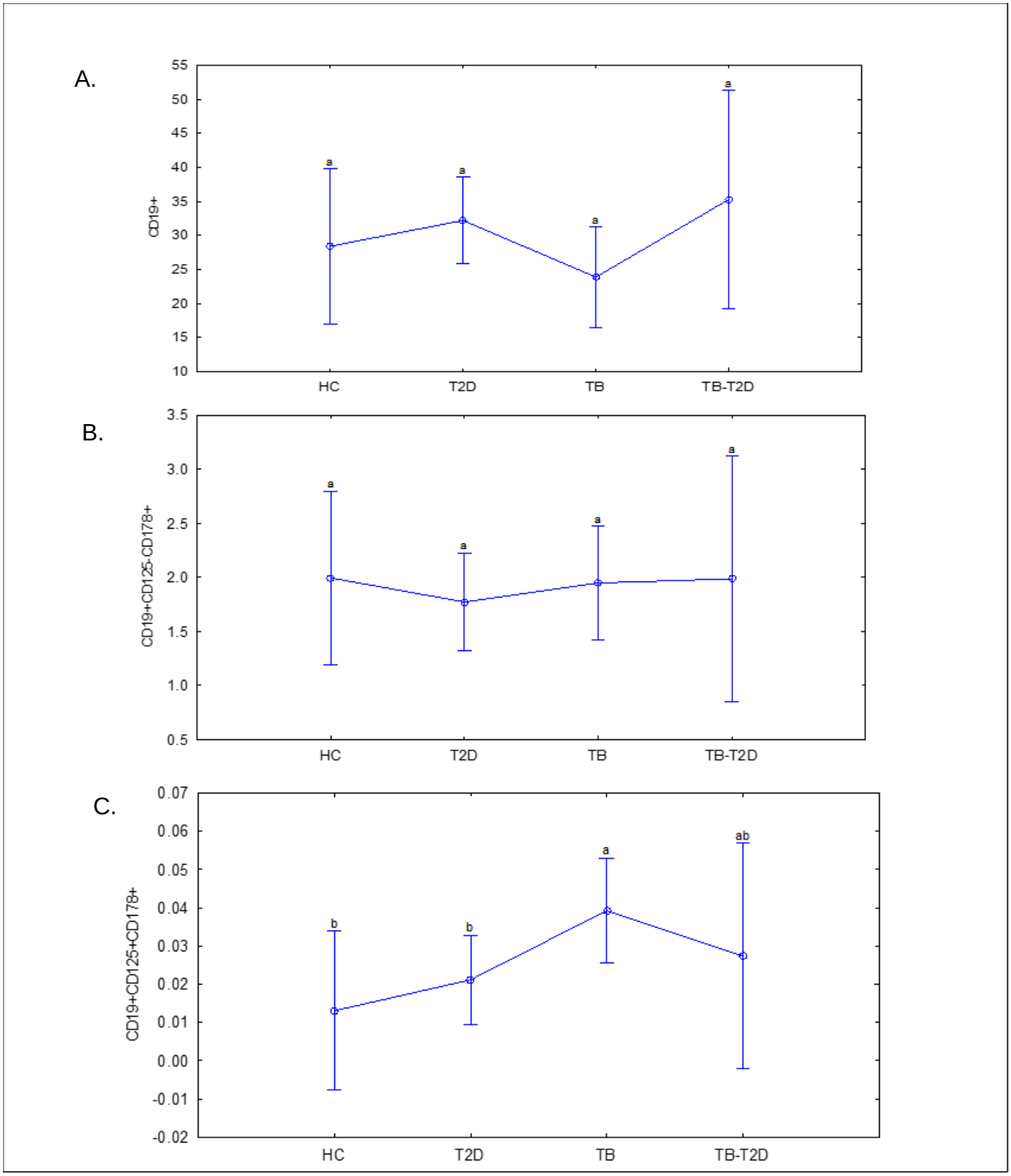

Figure 4.3: The frequencies of B cells expressing CD125$^+$ and CD178$^+$. Line graphs - A) Naïve B cells, B) Naïve B cells expressing CD125$^-$ and CD178$^+$ and C) Naïve B cells expressing CD125$^+$ and CD178$^+$: in HCs and patients with T2D, TB and TB-T2D before anti-TB treatment. The CD19$^+$ gate was obtained from gating on the CD3$^-$ population of the lymphocyte population. Cell frequency is expressed in (%). Statistical analysis was done using ANOVA with a Fisher LSD post hoc test to compare the differences amongst groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference among groups. Groups sharing letter are not significant difference from each other.

4.5 The expression of Killer B cells is higher in TB patients compared to HCs

Killer B cells are defined by the phenotype $CD19^{+}CD5^{+}IgM^{+}CD38^{+}CD125^{-}CD178^{+}$ and $CD19^{+}CD5^{+}IgM^{+}CD38^{+}CD125^{+}CD178^{+}$ and their killer function defined through the expression of $CD125^{-}CD178^{+}$ or $CD125^{+}CD178^{+}$ specifically. The expression of killer B cells is phenotype specific (Figure 4.4 A and B). In the absence of $CD125^{-}$ (Figure 4.5A) the expression of the killer B cells does not change within the four study groups. In the presence of both apoptotic markers (Figure 4.4B) the killer B cells decreases in patients with T2D.

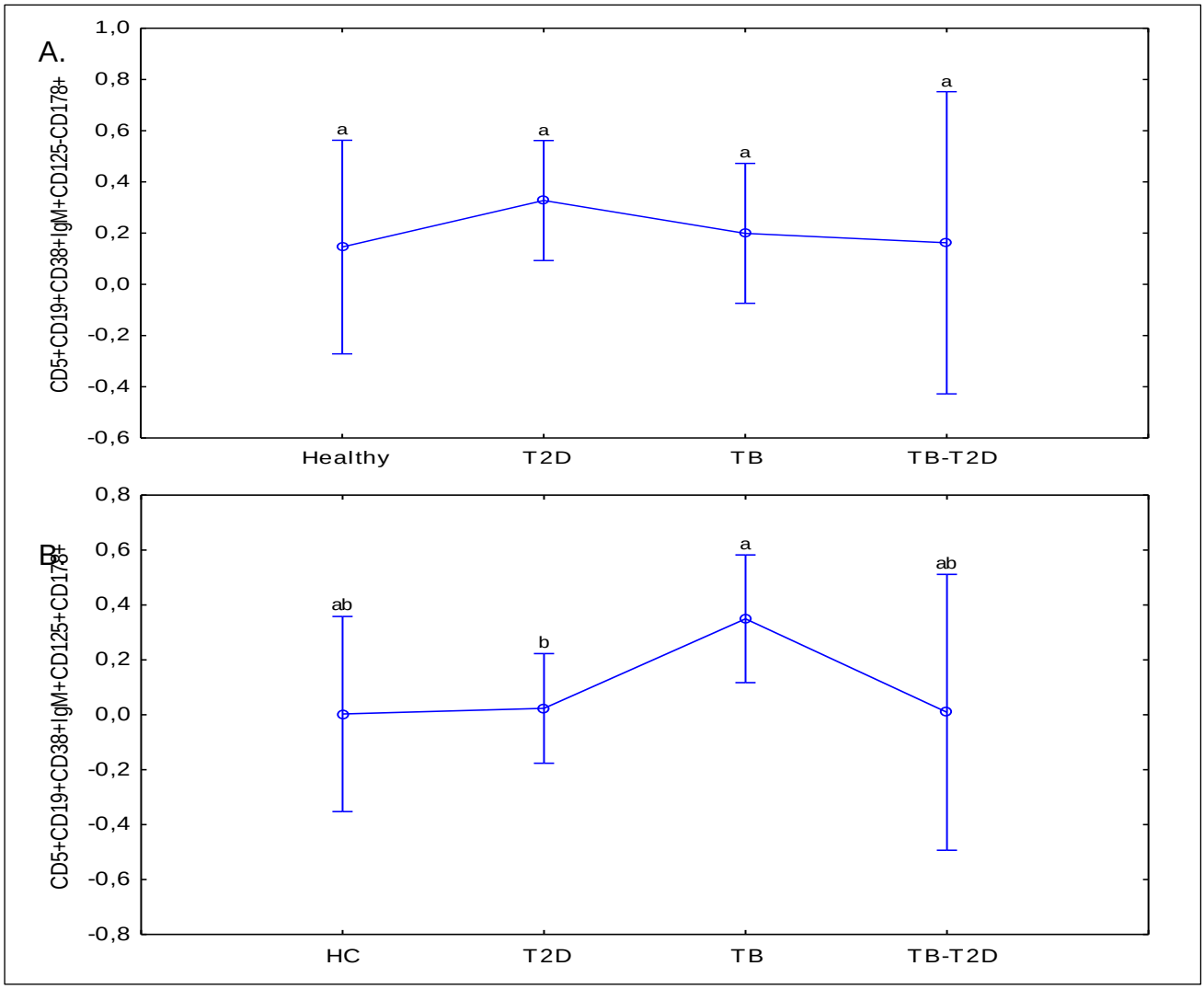

Figure 4.4: The frequencies of Killer B cells in HC, T2D, TB and TB-T2D at the initiation of TB treatment. The phenotype of killer B cells is defined by $CD19^{+}CD5^{+}IgM^{+}CD38^{+}CD125^{-}CD178^{+}$ and $CD19^{+}CD5^{+}IgM^{+}CD38^{+}CD125^{+}CD178^{+}$ and the d frequencies (%) in HC (n=6), T2D (n=19), TB (n=14) and TB-T2D (n=3) before anti-TB treatment. The $CD19^{+}$ gate was obtained from gating on the $CD3^{-}$ population of the lymphocyte population. Statistical analysis was done using ANOVA with a Fisher LSD post hoc test to compare the differences between groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference among groups. Groups sharing letter are not significant difference from each other**.**

4.6 The expression of regulatory B cells is higher in TB patients at baseline and lower in patients with T2D

Regulatory immune cells are associated with an immunosuppressive function through the secretion of anti-inflammatory cytokines IL-10, TNF-β and IL-35 responsible for immune tolerance proving to be beneficial to the host, however in the presence of disease this function is altered (Medzhitov, 2008; Vered et al., 2013). To better understand the regulatory role of B cells in TB patients with and without T2D, we investigated the different Breg phenotypes as defined by Blair *et al.* whose mechanism was assessed through their effect on autoimmune disease. Increased frequencies of $CD19^{+}CD5^{+}CD24^{hi}CD38^{hi}$ Bregs are found in TB patients (Figure 4.4B) compared to T2D ($p=0.08$). The expression of Bregs at the initiation of treatments response seems to be driven by TB where their frequencies are increased in relation to patients with T2D (T2D and TB-T2D patients). Although no statistical significance was observed in $CD5^{+}CD19^{+}CD24^{+}CD38^{+}$ a similar trend was observed as in Figure (4.4B).

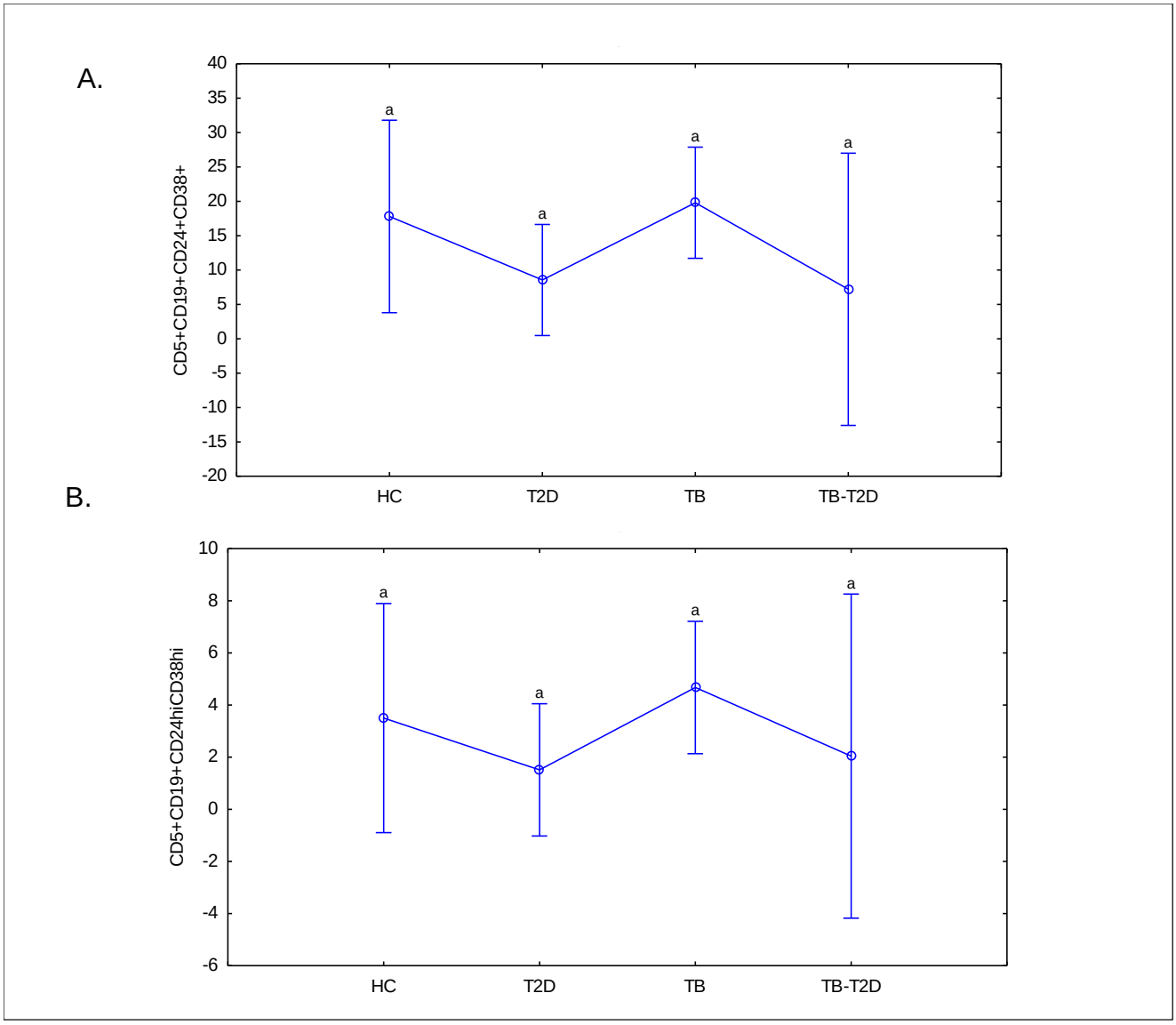

Figure 4.5: The frequencies of Regulatory B cells in HCs and T2D, TB and TB-T2D patients at the initiation of TB treatment. The frequencies (%) of Bregs; $CD19^{+}CD5^{+}CD24^{+}CD38^{+}$ and $CD19^{+}CD5^{+}CD24^{hi}CD38^{hi}$ were measured in HCs (n=6) and T2D (n=19), TB (n=14) and TB-T2D (n=3) patients before anti-TB treatment. The $CD19^{+}$ gate was obtained from gating on the $CD3^{-}$ population of the lymphocyte population. Statistical analysis was done using Statistica using an ANOVA with a Fisher LSD post hoc test to compare the differences among groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference. Groups sharing letter are not significant difference from each other.

4.7 The frequency of B cells and Bregs expressing apoptotic markers CD125 and CD178 change during the first two months into anti-TB treatment

Sputum smear conversion of TB patients undergoing anti-TB treatment commonly occurs at two months of treatment (Azarkar et al., 2016). The two-month sputum smear conversion is a useful predictor of anti-TB treatment outcomes (Kuaban et al., 2009; Singla et al., 2003) and delay in sputum smear conversion is associated with increased bacterial load and comorbidities such as T2D which delays sputum

conversion (Socorro Nantua Evangelista et al., 2018). We therefore investigated B cell frequencies in TB patients before and at months two of anti-TB treatment. B cells expressing apoptotic markers ($CD19^{+}CD125^{+}CD178^{+}$) decrease by two months of treatment (p=0.07) (Figure 4.6A). the frequency of Killer B cells, on the other hand ($CD5^{+}CD19^{+}CD38^{+}IgM^{+}CD125^{-}CD178^{+}$) increase from baseline to month to of anti-TB treatment (Figure 4.6B).

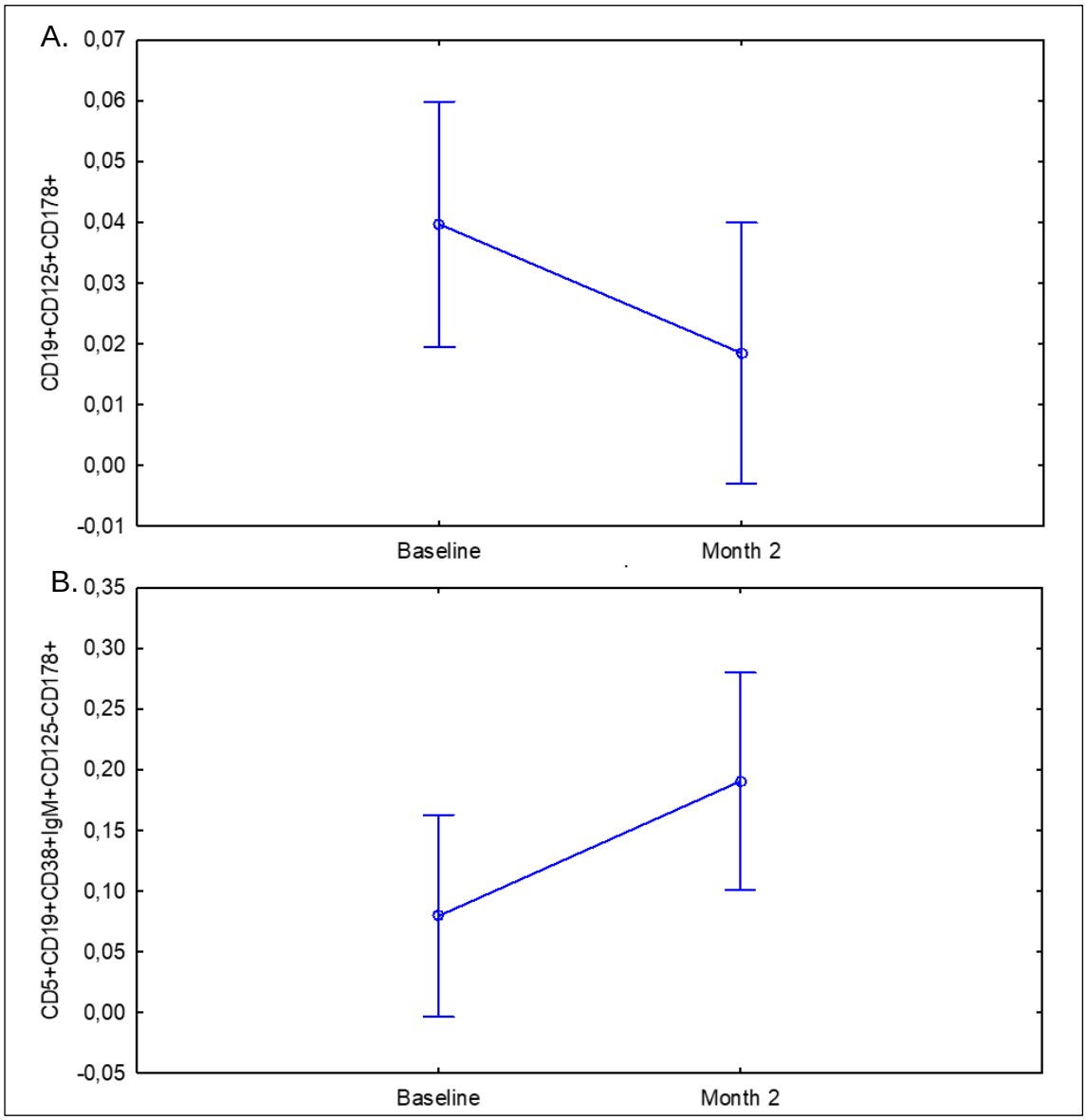

Figure 4.6: The frequency of B cells expressing $CD125^{+}$ and $CD178^{+}$ and $CD19^{+}CD5^{+}IgM^{+}CD38^{+}CD125^{-}CD178^{+}$ at the initiation and month two into anti-TB treatment. (A) The frequency (%) of B cells expressing $CD125^{+}$ and $CD178^{+}$ (p=0.07) and (B) the frequency (%) of killer B cells is defined by $CD19^{+}CD5^{+}IgM^{+}CD38^{+}CD125^{-}CD178^{+}$ (p=0.05) TB (n=14) at baseline and TB (n=12) two months into anti-TB treatment. Statistical analysis was done using ANOVA with a Fisher LSD post hoc test to compare the differences between groups. Results are represented as LS means with 95% confidence intervals. Significant letters are used to indicate significant difference among groups. Groups sharing letter are not significant difference from each other.

4. Conclusion

There is a decline in T-cell specific immune responses in *Mtb* infected individuals, demonstrated by the decline in functional T cells in these patients in comparison to HCs. Interestingly, TB-T2D patients were observed to have slightly lower activated T-cell frequencies compared to TB patients, suggesting an augmented immune cell dysfunction associated with T2D. The low memory B cell frequencies in TB patients are suggestive of a dysfunctional memory B cell response compared to the memory immune response of HCs and T2D patients. Naïve B cells expressing apoptotic markers $CD125^{+}$ and $CD178^{+}$ decrease two months into anti-TB treatment while the phenotypic killer B cells increase at month two. There were no statistically significant differences between the clinical groups for regulatory killer B cells.

Chapter 5

5.1 Cytokine profile of TB patients with and without T2D

Patients with T2D infected with *Mtb* are at an increased risk of developing TB and when they develop TB, they present with a greater severity to the disease. The immunological mechanisms driving the increased susceptibility are still largely unknown and there is still a need to further characterize the immune responses in these patients to better understand the cause for the increased susceptibility. The increased susceptibility is suspected to be due to the defects in the phagocytic ability of monocytes in patients with T2D. Phagocytosis is heavily dependent on opsonization, opsonins bind to the surface of the pathogen, increasing the number of binding sites that the phagocytic receptors can bind to, enhancing the efficiency of phagocytic cells. In patients with T2D, the functional efficacy of opsonin receptors namely FcγR and the complement receptor expressed on phagocytic cells such macrophages, neutrophils and DCs is altered (Restrepo et al., 2014). There is also a delay in the entry of *Mtb* into monocytes in T2D patients (Restrepo et al., 2014). These defects could be responsible for the increased susceptibility of T2D patients to TB.

In this study, patients with TB were followed up to month two of TB treatment. Month two is a crucial timepoint for patients undergoing TB treatment because sputum smear conversion occurs during this timepoint (Azarkar et al., 2016). A delay in sputum smear conversion is associated with an increase in bacterial load and in some instances an indicator of treatment failure and resistance to TB treatment (Singla et al., 2003; Kuaban et al., 2009; Unsematham and Kateruttanakul, 2013). Increased mycobacterial load is also associated with disease comorbidities such as T2D (do Socorro Nantua Evangelista et al., 2018). TB patients with T2D also present with a high *Mtb* load, delayed sputum smear conversion and are more likely to fail TB treatment (Stevenson et al., 2007; Jeon and Murray, 2008b; Riza et al., 2014). However, TB-T2D patients from this study were sputum culture negative at month 2 and the patients were not followed up until the end of treatment. In this study we could not validate the reported treatment responses of TB-T2D patients.

The immune system is a complex system and is carefully orchestrated by the cytokines produces by all cells of the body. Cytokines are secreted by immune cells in response to an antigen and they play an essential role in the recruitment and activation of immune cells and they also facilitate cellular processes such as cell division and cell growth. Chemokines are a subset of cytokines known as chemoattractant cytokines responsible for the recruitment of immune cells from the peripheral into the tissue. They achieve this function by binding to their receptors expressed on the cell membrane of all leukocytes. Chemokines are grouped according to their structure and function with the CC and CXC subgroups consisting of most of the chemokines. CC and CXC ligands are expressed in the bone marrow and thymus revealing their role in the development, maturation and differentiation of immune cells (Liu et al., 2006; Misslitz et al., 2004; Serbina and Pamer, 2006).

In chapter 3, heatmaps were generated to cluster serum host immunological markers based on their mean concentration for each patient group. In cluster 1, the concentration of MDC and MIP-1β, belonging to the CC family, and CXCL9/MIG, belonging to the CXC family, are increased in HCs in comparison to T2D and TB patients at baseline. This finding suggests that TB disease caused by *Mtb* and T2D driven by hyperglycaemia and insulin resistance may contribute to impaired chemotaxis of immune cells to lung during infection. Although no statistical significance was found in the TB-T2D group, they had a similar trend in mean concentrations as the TB and T2D group. MDC concentrations are directly correlated to monocyte frequencies (Godiska et al., 1997). This further supports our claim of defects in chemotactic function at the early stage of TB disease, as the MDC concentration increases in patients with TB (TB and TB-T2D group) at month two of treatment as represented in Figure 3.8.

TB patients with T2D have been reported to present with a greater extent of disease severity in comparison to TB patients without T2D (Jeon and Murray, 2008b). MMP-3 is a protein involved in the degradation and the breakdown of the extracellular matrix facilitating tissue remodelling, cell migration and recruitment and by so doing, modulating chemokine and cytokine signalling (Page-McCaw et al., 2007). MMP-3 also plays a role in tissue repair in diseases where tissue damaged occurred as a result of chronic inflammation (Ugarte-Gil et al., 2013). Their concentration increases in relation to TB treatment responses in the TB-T2D group. TB disease is associated

with lung tissue damage and anti-TB therapy is known repair tissue damage. We hypothesize that the increase of MMP-3 concentrations in the TB-T2D group during treatment is suggesting the body is trying to repair the greater tissue destruction that has been reported to be present in these patients. It is most likely due to a positive feedback mechanism, whereby the body produces more MMP-3 to compensate for tissue damage. In latently infected hosts with T2D, T cell activation is delayed while Th1 and Th17 responses increase in TB-T2D patients in response to an *Mtb* antigen (Restrepo and Schlesinger, 2014). The production of CXCL9/MIG, MDC and MIP-1β are T cell dependent and the reduction in the concentration of these chemokines highlights the impairment of T cell responses in TB and T2D patients. While in the TB-T2D group the concentration of CXCL9/MIG and MIP-1β is low at the initiation of TB treatment and increases at month two of TB treatment. *Mtb*-specific T cell responses are essential for macrophage activation, however at baseline in TB patients the concentration of MIP-1β and MDC which are responsible for monocyte recruitment are decreased in these patients. MIP-1β has been used in TB biomarker to distinguish between patients infected with TB and patients with TB disease (Yao et al., 2017). TB patients have a higher *Mtb* load than latently infected individuals. Although, this was not the focus in our study, it correlates with our finding where there are increased concentrations in TB patients at baseline.

Th1 immune responses form part of cell-mediated immunity and the activation of macrophages. Macrophage frequencies are altered in TB-T2D patients (Lopez-Lopez et al., 2018) and their phagocytic ability is impaired (Raposo-García et al., 2017). Due to the chronic inflammation present in TB-T2D, there is a decline in Th1 immune responses, and this could explain the decreased concentrations of sIL-4R in T2D and TB-T2D patients. A finding by Chang *et al.* 2012 can further justify our speculation where they showed that IL-4 can regulate glucose and lipid metabolism. T2D patients are characterized to have high glucose and lipid concentrations and T2D patients in this study had lower concentrations in comparison to HC.

sIL-4R and complement C5a had a similar effect in the different patient group and form part of cluster 3. In the introduction section of this chapter, we highlighted role of the complement system in the context of its receptors and their role in opsonisation. The defects in opsonisation were correlated to the defects in complement receptors and phagocytosis in patients with T2D (Restrepo et al., 2014). The concentration of

complement C5a in our study was lower in TB patients with T2D in comparison to TB patients without T2D. This can further validate the role of the complement system during opsonisation, where TB-T2D patients phagocytic ability of cells is impaired resulting in an increase in *Mtb* load associated with disease severity in these patients.

Eotaxin is an eosinophil specific chemoattractant responsible for the recruitment of eosinophils to the site inflammation. Eosinophils can generate and store eosinophil specific toxins in granules and are referred to as eosinophil granular proteins, these proteins are increased in patients with TB (Moideen et al., 2018) and are associated with ROS generation. We found eotaxin concentrations to be lower in TB patients compared to HCs. Furthermore, no change in serum concentration was observed in TB patients during TB treatment. Although literature reports high eotaxin concentrations in TB patients, our results are contradictory to what is commonly reported on eotaxin in the context of TB. However, a study done by (Choi et al., 2016) confirms our result as they also observed low concentrations of eotaxin in TB patients at baseline. Interestingly, their study was also conducted on a South African population. They also observed a treatment response in TB patients whereby the eotaxin concentrations increased with TB treatment. We did not observe this in our TB patients, but their study participants were triple our numbers and maybe it is due to our low statistical power that were did not observed a treatment response in TB patients.

Apo AI and Apo CIII are protein components of HDL which are involved in glucose metabolism through the secretion of insulin from pancreatic β cells (Drew et al., 2009). Apolipoproteins are involved in the translocation of GLUT4 to the cell surface. Apo AI increases glucose disposal in skeleton muscle supporting the role of HDL in reducing insulin resistance (Tang et al., 2019). Apo CIII is also associated with insulin resistance, inflammation and β cell failure in T2D (Åvall et al., 2015). The concentration of Apo AI and Apo CIII were lower in TB patients with T2D in comparison to TB patients without T2D. TB patients are associated with low HDL concentrations (Akpovi et al., 2013; Gebremicael et al., 2017). However, one of the limitations in this study were, we did not measure the lipid concentrations of TB patients and we therefore cannot speculate on whether their lipid profile would correlate with the observed high concentrations of Apo AI and Apo CIII. The concentrations of Apo CIII decrease in TB patients at month two of TB treatment, although not statistically significant, their

concentration increases in the TB-T2D group. Apo CIII is corelated with the mechanistic features driving T2D and hence why treatment does not seem to decrease their concentration.

High concentrations of Apo A1 and Apo CIII are associated with inflammation and correlate with inflammatory markers such as SAP and ER stress (Botteri et al., 2017; Wu et al., 2019). In this study the concentrations of Apo CIII was higher in TB patients compared to TB-T2D patients at baseline and both Apo A1 and Apo CIII significantly decrease from baseline to month two of treatment in TB patients only. The decrease in Apo AI and Apo CIII could be associated with inflammation as inflammation decreases during TB treatment as observed by the decline in inflammatory markers.

Alpha-1-antitrypsin (ATT) is correlated with poor lung function, failure to respond to anti-TB treatment and severity of TB disease (Levy et al., 2007; Almeida et al., 2009; de Melo et al., 2019). Our results correlate with previous findings, as ATT concentrations are higher in TB and TB-T2D patients at baseline and decrease two months into treatment. CRP and SAA are inflammatory markers and CRP is widely used as an indicator of inflammation. Inflammation is increased in TB and TB-T2D patients at baseline and the inflammation decreases two months into TB treatment.

5.2 B cell phenotyping in TB patients with and without T2D

B cells are the primary coordinators of the humoral immune response, however their immunological function within T2D remains poorly defined. It has been shown that in T2D patients exposed or infected with *Mtb* have altered frequencies of naïve and activated B cells (Kumar et al., 2015). Our group also found that the absolute number of B cells in TB-T2D patients was lower than that of TB patients (unpulished). This study therefore aimed to investigate known published phenotypes of B cells in relation to TB patients with T2D. One of the beneficial characteristics of the adaptive immune system is immunological memory. During TB disease this proves crucial in order to mount an effective and rapid immune response upon encountering *Mtb*. B cells can also utilize this function and we investigated the resting and activated memory B cell ratios.

HCs and TB-T2D patients at baseline expressed similar ratios between the resting and activated memory B cells with higher resting to activated memory B cell ratio in

T2D and TB patients although there are higher concentrations in resting memory B cells in T2D. The activated memory B cells are lower in T2D and TB patients at baseline. Activated memory cells are effective in regulating a fast and effective immune response. Memory B cells have two main functions, one they act as APCs and secondly, they secrete cytokines in response to an antigen. The low frequency of effector memory B cell observed in T2D patients in comparison to the resting memory B cells could result in the dysregulation of the above-mentioned functions resulting in an altered immune response.

Apoptosis is beneficially to the host as it removes potentially autoreactive immune cells from the peripheral and in tissues controlling the number of activated immune cells in response to a pathogen. The FasL-Fas pathway was previously associated with natural killer cells and $CD8^+$ but the Fas receptor is also expressed on activated B cells (Hahne et al., 1996; Schattner and Friedman, 1996).

Naïve B cells expressing the apoptotic phenotype of $CD125^+CD178^+$ are increased in TB patients and lower in HCs and T2D patients. FasL is highly expressed by $CD5^+$ B cells (Yang et al., 2013) and our group defined $CD19^+IgM^+$ as killer B cells (van Rensburg et al., 2017) and in this study the killer B cell phenotype was defined as $CD19^+CD5^+IgM^+CD38^+CD125^+CD178^+$. A similar response as the naïve B cells was observed with the killer B cell phenotype. However, the expression $CD19^+CD125^+CD178^+$ in TB patients decreases two months into anti-TB treatment while the killer B cells of TB patients increases at the two-month mark.

Bregs have immunosuppressive properties, a function they achieve through the secretion of anti-inflammatory cytokine IL-10. The frequency of Bregs is higher in TB patients at baseline compared to T2D patients. There were differences between the study groups in Bregs expressing the apoptotic phenotype at the initiation and at month of TB treatment. IL-10 serum concentrations although not correlated with the phenotypic data had an opposite effect where the levels were higher in TB patients and lower in T2D patients. This correlates with a finding by Winer *et al*. where the B cells of obese individuals linked to insulin resistance resulted in the decreased production of IL-10.

B cells have a high expression of MHC II on their cell surface allowing them to exercise their APC function where they present antigens to $CD4^+$ T cells. From the cytokine

data, the T cell responses were declined in T2D patients more especially the Th2 responses. CXCR5 a receptor for CXCL9/MIG is involved in B cell differentiation, activation and antibody production (Schaerli et al., 2000; Kim et al., 2001) and activation via the CXCR5 accelerates B cell immune responses (MacLeod et al., 2011). The serum concentrations of ligand CXCL9/MIG were decreased in T2D patients whose response is Th2 driven.

Conclusion

The immunological responses in TB, T2D and TB-T2D patients are dysregulated and improve at month two of TB treatment in TB and TB-T2D patients. Markers associated with cell proliferation, cell development, chemotactic function, granuloma formation and monocyte recruitment were dysregulated. Notably, Th2 responses the prime immune response during *Mtb* infection and regulators of inflammation are downregulated. Immunological analytes associated with disease severity, cell recruitment and opsonisation were also dysregulated in TB patients with T2D. The memory response, regulatory and apoptotic function through the expression of FasL and IL-5Rα in B cells is impaired in T2D patients and TB patients at baseline. There were limitations in this study as study participants differed between the cytokine profiling and phenotypic cohorts and hence there was direct correlation conducted. The TB-T2D patient numbers were extremely low in this study and hence there was a lack in statistical significance from the group.

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Appendix

INFORMATION DOCUMENT:

TANDEM – Concurrent Tuberculosis and Diabetes Mellitus: Unravelling the causal link and improving care

Stellenbosch University Ethics Committee project number:
IRB0005239 Protocol number: N13/05/064

Dear Participant,

We would like to ask you to take part in a study which investigates the link between TB and Diabetes. The study forms part of an international project funded by the European Union , which will investigate over 2000 participants in four different countries (South Africa, Indonesia, Romania and Peru). Your participation is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. We will explain the study to you. Please ask questions so that you understand clearly what this is about. You are under no obligation to give permission for this study. If you agree to take part, please read this form and sign the consent sheets at the end. Please tick off every box, if you agree. This study has been approved by the Health Research Ethics Committee (HREC) at Stellenbosch University (contact person Elvira Rohland at 021 938 9677 or elr@sun.ac.za) and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

The contact details of the investigator are as follows: Dr. Gerhard Walzl, Division of Medical Biochemistry, Stellenbosch University, Phone number: 021-9389158 or 0825923212.

What are TB and Diabetes?

Tuberculosis (TB) is a disease caused by a germ, and is spread by coughing. TB affects adults and children, and mostly causes disease in the lungs. Household contacts of TB patients and people infected with HIV are at increased risk of being infected with TB and getting TB disease. When someone comes into contact with a person who is ill from TB, they have a high risk of also getting infected with TB. Infection means that they have the TB germ in their body, but will not necessarily get ill (TB disease) from it. TB disease causes cough, breathing problems, weight loss and fever. Without treatment TB can be deadly, but with treatment you will usually get better. Many TB patients also have diabetes mellitus (DM), a disorder with how sugar is processed in your body. Early on diabetes has no symptoms, so many people do not know they have this condition. Screening DM among TB patients is not yet routinely performed. Also treating two conditions is not easy, TB drugs can temporarily worsen diabetes and, diabetes may worsen TB treatment outcome. Through this project we would like to understand more about the link between TB and DM and identify how to improve the diagnosis and treatment of people with TB and diabetes.

What is the purpose of the Study?

The purpose of this study is to answer a number of practical questions about how to diagnose DM in people with TB, how best to control blood sugar levels in people with TB and DM and to understand how these two diseases are related. Study participants diagnosed with TB will also be checked for DM and those with DM will be checked for TB. Then, if you are diagnosed as having both TB and DM, we will refer you to the respective clinics to treat both conditions and will follow you up until 1 year after you finish your TB treatment. For this study we will recruit 500 TB patients, 100 Diabetes patients and 100 healthy individuals.

Why have you been chosen to participate in this study?

You have been chosen to participate in this study, because you have been diagnosed with TB, Diabetes, HIV, or you are a healthy control participant.

What tests are we planning to do?

We anticipate that you will be in this study for 18 months (12 months after completing TB treatment). If you are a participant with Diabetes who does not have TB or if you are a healthy participant then we will only see you once during this study.

We will take you for a Chest X-ray at time of recruitment and if you have TB also end of treatment (usually 6 months) and at 12 months after completing TB treatment (18 months). For diabetic and HIV patients, we shall also do a sonar test of the blood vessels in your arm– only once if you have diabetes, and at day 0, month 12 and month 18 if you have HIV. We will collect the following body fluids on day 0 (recruitment), week 1,2, month 1,2,4,5,6,9,12,15 and 18. For some participants there will be fewer time points and fewer body fluids collected and you will be informed at the beginning of the study which time points points/body fluids will be left out):

- sputum (10ml or about two teaspoons full)
- blood (70ml or 14 teaspoons)
- urine (20ml or 4 teaspoons)

You will undergo a DM screening process. This involves a questionnaire and testing your blood and urine for sugar. For this purpose some of the blood we will collect and a urine sample will be sent to the lab. We also perform a finger prick test. Your sputum, blood, urine will also be tested for how your body responds to treatment. Some of the tests done on your specimen will include studying your genetic material, also called DNA or RNA. Our genes determine what we look like and sometimes what kind of diseases we may be susceptible to. During this study we will try to find genes that make us more susceptible to TB and diabetes. We will also determine your ancestry in other words how much of your genetic material is from white, black African, Asian, Khoi-San ancestors. After completion of TB treatment we would like to continue to check on your health regularly (every 3 months until 12 month after completion of TB treatment).

We will also ask your consent to do an HIV test on you. The HIV test results will be available immediately, and if the test is positive, we will confirm the result with another test. This second result will also be available immediately. Before we do the HIV test, the study nurse who will draw the blood will counsel you about the implications of this test result. After the test has been done and you know the result, we will counsel you again. This process is called “pre-and post test counselling”. It is important to know your HIV status as there is treatment available which can help you a lot although the treatment cannot cure HIV. We would refer you to the Infectious Diseases Clinic at Tygerberg Academic Hospital or another HIV clinic in your area

for care. At these clinics, good treatment for HIV infection (anti-retroviral therapy) is now available to those patients who need it.
If you have previously been diagnosed with HIV, we shall do a confirmation test for our records.

What are the implications of this study for you?

If you decide to take part in this study you will have to return to the clinic for follow up visits. Your participation in the study would be ended if you missed follow-up appointments.
You will not be paid to take part in this study. Although some remuneration for expenses occurred incl. transportation and loss of income will be given to you for your effort and time (approx. R 100).

What are the benefits to your taking part of this study?

No individual study results from this study will be available for participants, as this type of result does not offer a benefit to individuals at this stage. We do hope however that the results will benefit the communities that are affected by TB and Diabetes.

What are the potential risks of this study for you?

We will not perform genetic analysis of any underlying medical conditions except TB and Diabetes. We will not perform any analysis related to paternity testing. Therefore the outcome of the analysis has no implications for you or your family.

The only discomfort you may experience is from a pinprick in your finger and the blood draw. Both will be minor.

If you cannot produce enough sputum we will perform a procedure called ‘induced sputum’ collection. For this test you will be asked to breath in a salt solution through a nebulizer, similar to the ones used by asthmatics. Some people may develop transient breathing difficulties but we will have a trained nurse and the necessary medication on site to deal with this.

We may store some of your samples in a freezer for future testing if necessary. The blood and other body fluids will be stored for up to 10 years, but will only be used for this specific research project. Most of the samples will be sent to a laboratory in another country for some specialized tests since these tests are not available in South Africa. These laboratories will be part of this project. Should you decide to withdraw your consent at a later stage, you may ask for the destruction of your samples that were obtained from you earlier than this date. After 10 years your samples will either be destroyed or we will remove any link to your identity from the samples so that it can never be traced back to you.

How will your confidentiality be protected?

All information about you will be kept private and confidential. Your name will not accompany the material that we send away and will never be made public. Information of your genetic make up will not be made available to anyone. Members of the Research Ethics Committee may inspect the research records.

Whether you choose to participate in this study or not, will have no effect on your treatment whatsoever. If you would like to withdraw from this study, you may do so at any time point and this too will have no negative implications on your treatment.

Thank you for considering participation in this study.

Dr. Walzl and the study team.
Molecular Biology and Human Genetics, Department of Biomedical Sciences, Stellenbosch University and Tygerberg Hospital, Francie van Zijl Drive, Parow.
Phone 9389158 or 0825923212

INFORMED CONSENT FORM:
TANDEM

Please read, tick off each of the boxes and sign the form if you agree to take part in this study.

1. **I understand what this study is about and know how to contact the investigators if I want to.** ☐
2. **I understand that body fluids (blood, sputum, urine) will be collected from me/the participant and that tests incl. genetic analysis will be done.** ☐
3. **I understand that all the information given to the investigators and all test results will be kept private and confidential.** ☐
4. **I understand that I will not benefit financially from this study apart from being compensated for transport costs and loss of income on the days that I take part in the study.** ☐
5. **I understand that I am free to withdraw myself/the participant from this study I want to.** ☐
6. **I understand that if I refuse to take part in this study, that my/the participant's care will not be affected.** ☐
7. **The information was explained to me by:** _____

Name of participant: _____

Address of participant: _____

Witness name _____ **Witness signature:** _____

Date: _____

Tick the option you choose:

☐ I agree that my blood or tissue sample can be stored **indefinitely** after the project is completed but that it is anonymised with all possible links to my identity removed, and that the researchers may then use it for additional

research in this or a related field. Once my sample is anonymised, my rights to that sample are waivered. My sample may be shipped to another laboratory in SA or abroad to be used in other research projects in this or a related field

OR

☐ Please destroy my blood sample as soon as the current research project has been completed.

Signed at (*place*) ..... on (*date*) .....

....

Signature of participant **Signature of witness**

INFORMED CONSENT FORM- HIV TESTING:

TANDEM

1. I give my permission for the investigators to do a HIV test on me/the participant. ☐

2. I have received pre-test counseling, understand what HIV is about, and realize the implications of this test for me/the participant. ☐

3. I understand that the investigators will perform a confirmatory test after a positive first test and that they will inform me of the final test result during the first visit. ☐

4. I understand that if I refuse permission for HIV testing, my/the participant's care not be affected. ☐

Name of participant:	
Address of participant:	
Signature of participant/legal guardian	
Name of person giving consent	
Capacity in which consent is given (i.e. self or legal guardian)	
Witness name	
Witness signature:	
Date:	

Post HIV test counseling was offered to me/the participant:

Name:	
Date:	
Signature:	

DECLARATION BY RESEARCHER OR REPRESENTATIVE OR RESEARCHER: TANDEM

I, ……..(name)

in my capacity as……(researcher/researcher representative) declare that:

1. The information in this document was explained to …… (name of the participant/representative).

2. I have given him/her the opportunity to ask further questions regarding the study and aspects of participation.

3. The information was given in ……(language) and no translator was used.

Signed:	
Place:	
Date:	
Witness:	